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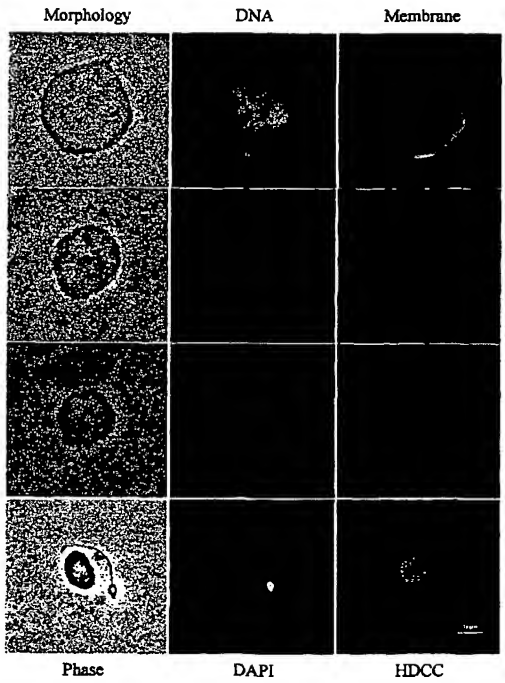

Human sperm dispersion, genomic reprogramming and reassambly of nuclear envelope.

(57) Abstract: The present invention relates to a method of sperm permeabilisation comprising: (a) washing a sample containing sperm in a sperm nuclear extraction buffer (SNEB) which includes one or more metal chelating agents and which is substantially free of non-ionic detergents and divalent cations; (b) contacting the sperm with one or more permeabilising agents; and (c) optionally separating the sperm tail from the sperm head; and to a method of sperm transformation which method comprises incubating a sample of sperm or sperm nuclei in one or more cell-free extracts. Also described are extracts and buffers for use in these methods, methods of preparing the extracts and uses of the transformed sperm nuclei.

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Manipulation of spermatozoa

The present invention relates to the preparation of sperm nuclei for analysis or interaction with other cells or media. More particularly, the invention relates to a method of sperm nuclei permeabilisation, to a method of transforming sperm nuclei to a state capable of fusing with the nuclei of other cells and to those fusion methods. The invention also relates to media for use in said method of transformation and methods for the preparation of such media.

A few decades ago, while the global human population was increasing at an alarming rate, family planning and birth control measures were encouraged throughout the world, especially in the underdeveloped nations. These measures were effectively implemented with the help of hormone-based female contraceptives and occasionally by male vasectomy. While public awareness of overpopulation, reproductive health and birth control measures has increased considerably in recent years, several unforeseen and yet serious problems have emerged. Currently, the ability of human couples to reproduce is on the decline. These trends appear to coincide with a sharp drop in the mean sperm count of fertile men from 113 million per ml in 1940 to 66 million per ml in 1990. About 25% of the failed fertilisation or conception could be accounted for by the variety of defects in human spermatozoa. The overall increase in infertility or subfertility in humans could be related to demographic changes including prolonged exposure to industrial pollution, lifestyle factors and an increase in the age of couples willing to reproduce.

Not too long ago, the social burdens of infertility, especially in the underdeveloped nations, were invariably carried by women. However, a few important breakthroughs in the last 25 years have led to the conclusion that male and female infertility occur in almost equal proportions in human populations. The causes of infertility are diverse, some are well established, some are controversial. Despite extensive diagnostic testing for azoospermia, for obstructive or unobstructive-oligospermia, ovulatory dysfunction, inflammatory tubal disease, endometriosis and varicocele, the etiology of at least 10% of the human infertility is unknown. Conventional physical parameters such as the measurements of volume of the semen, sperm concentration, morphology and motility used for evaluating the semen quality appears to be insufficient for the diagnosis of male infertility.

Much of male infertility could be accounted for by functionally defective spermatozoa (cell biological) and aberrant spermatogenesis (genetic). About 25-40% of infertile men have high levels of reactive oxygen species (ROS) in the semen leukocytes and in the spermatozoa. These superoxides are generated by hyperactivation of oxidative metabolic pathways in response to the increased cellular levels of reduced cofactor NADPH and H_2O_2. In addition to peroxidative damage of the plasma membrane which affects motility, capacitation and fertility of the sperm cells, the ROS also induces extensive damage and cross-linking of the sperm chromosomal DNA (Twigg, J.P. et al., Hum. Reprod. 13: 429-436, 1998). Such oxidative DNA damage of the spermatozoa has been strongly correlated with childhood cancers.

It should be noted that herein the terms sperm and spermatozoa are used inter-changeably.

Currently, the extent of sperm DNA damage is measured by autoradiography of radioactively labelled nicked DNA, by alkaline gel electrophoresis or by a single-cell 'comet assay' where the alkali-denatured damaged DNA migrates away from the sperm head in the gel generating a smear which appears like a comet. In addition to its semi-quantitative nature, the comet assay provides no information on whether genes vital for fertilisation and embryogenesis are disrupted by ROS in infertile patients. Given the unique organisation of the genome in human spermatozoa, one can not assume that even 50-70% of the DNA damage would affect all the genes (<5-10% of the total genome) needed for fertilisation and development. Moreover, much of the damage could be repaired by highly efficient DNA-repair mechanisms operating in the egg (Twigg, J.P. et al., Hum. Reprod. 13: 1864-1871, 1998).

The chromosomal abnormalities in male infertility include aneuploid (47,XYY and 47, XXY, Klinefelter's syndrome), X- or Y-autosome translocations and deletion of the Y chromosome. A detailed cytogenetic survey of azoospermic and oligospermic men revealed a strong correlation between infertility and abnormalities of the sex chromosome Y. Such abnormalities are represented by extensive deletions in the long arm of the chromosome Y (Yq11). Since chromosome Y is the smallest (2% of the haploid genome) of the 24 human chromosomes, a comprehensive physical map of the chromosome and >100 STSs became readily available. This information facilitated further analysis of microdeletions in azoospermia/oligospermia and resulted in the identification of three loci AZFa, AZFb and AZFc containing a number of genes which are specifically expressed in the testis for normal spermatogenesis and are called 'male factors' (Vogt, P.H., Mol. Hum. Reprod. 4: 739-744, 1998). Some candidate genes at Yq11

definitively shown to regulate male fertility have been identified. Most thoroughly studied candidate genes for male infertility are DFFRY(Y chromosome RNA recognition motif) at AZFb locus and DAZ (deleted in azoospermia) located at AZFc. Although DAZ and DFFRY belong to different gene families, they have remarkable functional similarities.

An *in vitro* system for analysis of human sperm chromosomes is urgently needed for understanding the effect of DNA damage and sperm chromosomal abnormalities on male infertility. Currently, this is accomplished by microinjection of human sperm into hamster eggs; only a few laboratories in the world perform this test. Unlike hamster eggs microinjected with sperm, the methods described herein are inexpensive, very rapid and do not require animals. Therefore, a large number of patients can be assessed and counselled within a short period prior to ICSI or other fertility treatments.

In addition to the investigation on male infertility, the methods can be widely applied in "Genetic risk assessment" and 'Occupational monitoring'. A significant number of people (1 in 200) in the human population are carriers of genetic defects which are manifested at the chromosomal level. These genetic lesions in the germ-lines arise more frequently among industrial workers exposed to ionizing radiation or radiomimetic agents and genotoxic environmental chemicals including >30% licensed pesticides. It is envisaged that many government organisations and companies would be keen to use a system to monitor the chromosomes of workers exposed to potentially hazardous agents.

As well as diagnostic applications, the technology of the present invention provides a new method of *in vitro*

fertilisation (IVF). Currently, one in five couples have difficulties with reproduction or are unable to conceive without medical help. Intracytoplasmic sperm injection (ICSI) is the most modern, effective treatment of infertility and has revolutionized the management of infertility cases in IVF clinics. In the ICSI techniques, a sperm is injected into the cytoplasm of an oocyte (for a review, see Tesarik and Mendoza, BioEssays [1999], 21, 791-801). Before the advent of ICSI, couples declared infertile because of a male factor were probably unlikely to have children. While ICSI continues to be a key therapeutic measure, the possible genetic risks of passing on damaged genes and defective chromosomes of the father to the embryo, resulting in first trimester pregnancy losses, congenital abnormalities, premature child birth and early childhood cancers are of serious concern. Unfortunately, the negative impact of ICSI on the genetic constitution of a population has never been evaluated by animal experimentation. Such studies in humans are impractical, if not impossible, primarily due to ethical considerations.

The sperm-egg fusion experiments proposed here could be optimised to establish the genetic risks of infertile males prior to fertilisation by sperm-somatic cell fusion and subsequent cytogenetic analysis of metaphase chromosomes.

The karyotyping of a significant number of sperm chromosomes by microinjection of zona-free hamster eggs with human spermatozoa and subsequent cytogenetic analysis is restricted to a few laboratories in the world. Pre-implantation diagnosis is restricted to a very limited number of diagnostic laboratories. The present invention also offers an alternative to ICSI which significantly reduces the possibility of passing

on damaged genes and defective chromosomes to the eggs. This is because the egg ooplasm from high age-group women exhibits reduced competence in the process of DNA repair during fertilisation and blastocyst development. The *in vitro* repair of damaged genes of the infertile sperm in cell-free extracts and subsequent micro-injection of such sperm nuclei to the oocyte could increase the efficiency of implantation and thereby might increase the chances of development of the embryo to term.

The technologies for fusion of two somatic cells by viral glycoproteins or chemicals (polyethylene glycol) or by transient electrical pulses have been developed over the last 20-30 years and are routinely used for producing somatic hybridomas or in a variety of cell biological and molecular research. However, existing technologies are insufficient to carry out this kind of fusion of sperm with somatic cells or with oocytes. This is because the fundamental necessities for *in vitro* cell fusion are that participating cells have similar morphology and membrane bilayer structures. Unlike somatic cells or oocytes, the mammalian spermatozoa are unique germ cells. For example, a) the human sperm is about one sixth the size of normal cells (for instance hepatocytes) and about one-fortieth the size of a mature human oocyte; b) unlike any other cells, the chromosomes in the sperm are super-condensed (almost crystallized) by special proteins only produced by males in the testis; and c) spermatozoa can neither divide nor transcribe their genome (no visible chromosomes by staining). These characteristics, despite rapid advancement of biotechnology in the past decade, have prevented cell fusion as well as hindering genetic or molecular research involving mammalian spermatozoa.

An especially challenging aspect of sperm- somatic cell

or oocyte fusion research is therefore transformation of the spermatozoa to cells which are morphologically and structurally similar to those of somatic cells or oocytes or at least similar enough to fuse with these cell types *in vitro*.

There are three kinds of system for sperm dispersion and nuclear assembly reported in the literature, relating to *Xenopus* (frog)oocyte (Lohka, M.J and Masui, Y. J. Cell Biol. 98: 1222-1230, 1984), sea urchin egg (Collas,P and Poccia, D. Therogenology, 49: 67-81, 1998) and fruit fly (*Drosophila*) embryo (Berrios, M and Avillion, A.A. Exp. Cell Res. 191: 64-70, 1990) extracts. These extracts prepared by releasing materials from mature eggs or fruit fly embryos by high speed ultra centrifugation, are capable of dispersing corresponding sperm nuclei (frog, sea urchin or fruit fly) and have been used to demonstrate sperm nuclear chromatin remodelling and nuclear envelope assembly. A comparable homologous system for human and other mammalian sperm dispersion and nuclear assembly has not been reported.

A method is described in WO95/21860 to transform the human sperm to individual chromosomes using cell-free extracts. The cells used in those studies were human somatic cancer cells (HeLa). As discussed above, sperm chromosomes are normally present in a highly condensed state and one important aspect of preparing sperm for fusion to other cells is to decondense the chromosomes. This patent application proposed a method for decondensation of sperm chromatin and/or chromosomes. However this system has a number of serious disadvantages. Firstly, the human sperm dispersion (decondensation) was never uniform because, as discussed below, the methods used to prepare sperm nuclei were inadequate.

Secondly, the sperm dispersion in this system cannot be controlled, therefore, the chromosomes of the dispersed nucleus in the absence of a boundary (envelope) start breaking down within a short period of time. This is a serious problem in many applications which require the sperm to form a normal nucleus with intact chromosomes and rigid nuclear envelope (membrane).

Furthermore, it is highly desirable to be able to control the size of the transformed sperm nucleus (with envelope) as different sizes of transformed sperm nuclei can be used for various purposes. For example, a relatively large nucleus is required for fusion with an oocyte, a smaller nucleus for fusion with somatic cells and a very small nucleus for Intracytoplasmic sperm injection (ICSI). For practical purposes, it would be highly advantageous if transformed sperm nuclei, once assembled, could be stored in liquid Nitrogen (-176°C) or at -70°C. This is impossible with the methods described for sperm decondensation using HeLa cell-free extracts (WO 95/21860).

A protocol for *in vitro* mammalian sperm nuclear transformation has now been devised. For example, it has been shown that mature mouse or human sperm can be transformed into a nucleus capable of fusion with other cell types by incubating the sperm in cell free extracts from embryonic stem cells or developing embryos.

The present inventor has now devised a series of techniques for preparing sperm nuclei from spermatozoa and transforming the sperm nucleus morphologically to a form which can fuse with somatic cells, oocytes or be injected into oocytes.

First is described a technique for preparing sperm, more particularly for permeabilising sperm nuclei. Thus in a

first aspect, the present invention provides a method of sperm cell permeabilisation which comprises,

(a) washing a sample containing spermatozoa in a sperm nuclear extraction buffer (SNEB) which includes one or more metal chelating agents (e.g. EDTA/EGTA or Bathophenanthroline sulphonate) and which is substantially free of non-ionic detergents (such as Triton X100) and divalent cations (such as Ca^{2+} or Mg^{2+}),

(b) contacting the spermatozoa with one or more permeabilising agents which create holes in the nuclear membrane, such as lysolecithin (L-α-lysophosphotidyl-choline) and/or digitonin, and

(c) optionally separating the sperm tail from the sperm head.

Prior to washing the spermatozoa in SNEB, the sperm sample e.g. human semen sample is preferably treated to separate active motile mature sperm for further steps, this may conveniently be achieved by application of the sample to a density gradient system such as a silica-based density gradient. The gradient purified sperm are preferably then washed in PBS or a similar buffer. If used, the gradient and PBS or like buffer should be substantially free of divalent agents as discussed above. A sperm sample obtained directly from the epididymis may conveniently first be filtered to remove tissue debris.

The permeabilisation step (b) will conveniently be carried out on ice for 10-30 minutes under regular mild agitation. Lysolecithin is a preferred permeabilising agent for use in step (b) and suitable concentrations vary from 50-400, preferably 100-250 μg/ml, in a particularly preferred embodiment spermatozoa are also contacted with a further permeabilising agent such as digitonin. Suitable concentrations for said further permeabilising agent are 25-500, preferably 50-300 μg/ml

in the SNEB.

The SNEB will preferably contain 5-30 mM e.g. 15-25 mM of HEPES-NaOH (pH 7.0), 50-300, eg 100-200 mM KCl, 100-400, e.g. 200-300 mM sucrose and 1-100, preferably 1-20, e.g. 3-10 mM of a chelating agent such as EDTA as well as one or more protease inhibitors such as antipain, aproteinin, leupeptin, chymostain and APMSF.

'Chelating' agents include molecules which are capable of forming covalent (more particularly co-ordinate bonds) with metal ions present in the sample or buffer. The amount of chelating agent present is preferably sufficient to sequester a significant proportion of the available divalent cations and provide an environment for sperm nucleus permeabilisation with a low concentration of divalent cationic metal ions, typically less than 200 μM, preferably less than 100 μM.

The permeabilisation step will preferably leave remnants of the original sperm nuclear membranes associated with the sperm nucleus i.e. holes are formed but parts of the nuclear membrane remain intact and *in situ*. Triton X100 at 0.25% to 2.0% solutions are normally recommended for human or animal cell nuclear isolations. However it has surprisingly been found that Triton X100 is not suitable for the methods of the present invention because it causes too great a disruption in the nuclear membrane of the sperm. For optimum sperm dispersion, discussed in more detail below, it is important that factors in the extracts used have access to the nuclear chromatin. However, re-assembly of the new nuclear envelope after dispersion has been found to be most efficient when remnants of the original sperm nuclear membrane remain associated with the dispersed nuclei. Thus, permeabilisation agents are selected which are more gentle and create holes in the nuclear membrane rather

than harsher reagents such as Triton. The SNEB in which the sperm are preferably suspended when in contact with the permeabilising agent(s) is substantially free of Triton, in other words it is completely absent or present at a concentration of no more than 0.1%, preferably no more than 0.05%.

Similarly, it is usually recommended that the buffer for nuclear preparation from human or non-human animal somatic cells should contain some divalent cationic agents, typically Ca^{2+}, Mg^{2+}, spermine or spermidine. It has been found that incorporation of Ca^{2+}, Mg^{2+} or other divalent cations in buffers used to isolate sperm nuclei does not allow successful nuclear dispersion. They are therefore omitted from PBS or any gradient system used to purify sperm from semen or epididymis and from SNEB. Functionally insignificant amounts of these cationic species may be present, e.g. less than 50-100 micromolar concentrations of divalent cations such as Ca^{2+} or Mg^{2+}. However, we find that human sperm in the absence of low concentrations of cationic agents may begin to aggregate (clumping) following permeabilisation. Therefore, SNEB for human sperm nuclear preparation preferably contains 0.1-1.0 mM, preferably 0.15-0.4 mM, e.g. 0.25 mM (each) of spermine and spermidine in addition to e.g. 3-5 mM chelating agents. Spermine and spermidine are used to avoid clumping of the nuclei. For mouse sperm preparation neither are necessary.

It has surprisingly been found that incorporation of e.g. 2.5-20, preferably 5-10 mM chelating agents in the SNEB dramatically improves the ability of the sperm nuclei to disperse.

In the above method steps (a) and (b) may be separate or simultaneous, e.g. the sperm sample may simply be suspended in the SNEB to which one or more

permeabilising agents have already or may subsequently be added.

A separate step for removal of the sperm tail is generally not required for preparation of human sperm nuclei. With human sperm, tail loss conveniently occurs during the permeabilisation step but some sperm may emerge from the preparatory (permeabilisation) method with tails intact. Generally, the sperm will then lose their tails during the incubations for dispersion/ reprogramming/reassembly.

In vivo transformation of the transcriptionally silent sperm head to a male pronucleus consists of a series of macromelecular events: sperm chromosome decondensation, release of protamines, DNA repair, chromosomal remodelling, assembly of organelles and a nuclear envelope around the reprogrammed haploid chromosomes. (Longo, F.J. Curr. Topics. Dev. Biol. 12: 149-184, 1978; Collas, P and Poccia, D. Theriogenology, 49: 67-81, 1998). All these events are accomplished by molecular chaperones, histones, non-histone structural proteins, DNA-repair enzymes and factors transiently accumulated in the egg ooplasm. Indeed, this maternal stockpile, in amphibians and flies, is so extensive that the zygote can replicate several thousand times independently of parental gene activation.

In humans and higher mammals with placenta, successful male pronuclear assembly and fertilisation are however largely determined by the quality of the egg cytoplasm (chromosome decondensation, DNA repair, demethylation and remodelling factors). The inventors have discovered that cell-free extracts incorporating all of these factors can be prepared and used in cell-free systems to transform sperm nuclei. Methods of preparing cell-free extracts for transforming sperm (nuclei) into a form

capable of fusing with nuclei of other cells are described herein. As a starting cell, any animal, preferably mammalian cell can be used. While differentiated somatic cells can be used, preferably the cells from which the extracts are derived are pleuripotent, more preferably ES cells or embryo cells. The use of ES or embryo cells is particularly preferred when the sperm nuclei is being transformed for fusion with an oocyte or microinjection into egg cytoplasm (as opposed to fusion with a somatic cell). The cells from which the extracts are generated need not be from the same species as the sperm which it is desired to transform.

It has been found that cell-free extracts in which the sperm (nuclei) can be incubated in order to achieve dispersion and reprogramming prior to fusion are preferably obtained from embryos or from embryonic stem cells. The use of cell-free extracts (preferably derived from embryos or embryonic stem cells) to disperse, reprogramme and reassemble sperm nuclei constitutes a further aspect of the present invention.

Thus, in a further aspect, the present invention provides a method of dispersion of a sperm nucleus and nuclear reprogramming and reassembly of the nuclear envelope of said sperm nucleus, which method comprises incubation of a sperm or sperm nucleus in one or more cell-free extracts, preferably derived from embryos or embryonic stem cells. The sperm/sperm nucleus is preferably permeabilised prior to incubation in the cell free extracts.

Herein, the preparation and use of cell-free extracts is described with reference to ES cells or embryos but extracts from other cell types may be prepared and used in an analogous manner.

As discussed above, when incubation to achieve dispersion/reprogramming/reassembly of the nuclear envelope begins, some of the sperm nuclei may still have their tails in tact, therefore reference herein to sperm 'nuclei' does not imply that the tail must be absent.

Mouse/human embryonic stem cells are pleuripotent (undifferentiated) and can be grown using special cultural conditions. The efficiency of the cell-free extracts derived from developing mouse embryos at 11-12 days of gestation is comparable to that of ES cell-free extracts.

A preferred incubation method comprises a two step protocol wherein the nuclei are incubated in first one then a second cell-free extract. Preferably at least one, more preferably both of these extracts are obtained from embryos or embryonic stem cells. Conveniently both of these extracts are obtained from embryos (conveniently 11-12 days post-coitus in mice or for humans, during the first trimester) or embryonic stem cells, preferably both extracts being from the same batch of preparations.

Thus typically, the first extract is prepared from a sample of embryos or ES cells. Typically, the embryos will first be dissected into small fragments. The samples are suspended in a buffer which is substantially free of divalent cations, more particularly of Mg^{2+} and Ca^{2+} and subjected to a gentle centrifugation step, typically 1,000-5,000 rpm for 5 to 25 mins, preferably 1,500 to 2,500 rpm for 10 to 20 mins. The resulting supernatant is referred to herein as extract A (or dispersion extract) and is used in the first step of the incubation methods described above. Extract A is responsible for both sperm dispersion and nuclear envelope assembly and thus must contain the materials

necessary for these processes. It is a novel and important feature of this extract that its components allow both dispersion and nuclear envelope assembly. While it could be envisaged that separate extracts or components are prepared and contacted with the sperm, it is important for optimum performance of the present invention that dispersion and reassembly happen substantially at the same time. In this way, formation of the enlarged sperm pronucleus can be controlled and breakdown of the chromatin avoided. Extract A thus provides a convenient mechanism for simultaneous dispersion and reassembly.

The pellet is resuspended in a buffer which may contain divalent cations such as Mg^{2+} at concentrations of 2.5-10mM, preferably 5mM and 2-10% e.g. 5% glycerol. The resulting suspension is then subjected to a moderate centrifugation step e.g. spun at 8,000-20,000 rpm for 15-45 mins, preferably 10,000-15,000 rpm for 25 to 35 mins. The resulting supernatant provides the extract used in the second incubation step and is referred to herein as extract B (or reprogramming extract). This extract B provides the materials necessary for reprogramming of the sperm nuclear chromatin.

The second supernatant is preferably dialysed prior to use in the incubation method and particularly preferably undergoes a further centrifugation step after dialysing e.g. 3,000-6,000 rpm for 10 to 20 mins, e.g. 3,800-4,800 rpm for 12 to 17 mins. The pellet produced is discarded. The above described methods of extract preparation constitute further aspects of the invention.

As discussed above, extract A should be free of divalent cations and extract B preferably contains Mg^{2+}. It may be possible to prepare extract B which does not contain Mg^{2+}, in which case, the two extracts may be brought

simultaneously or substantially simultaneously into contact with the sperm, e.g. by prior mixing.

Thus in a preferred embodiment, the present invention provides a method of sperm transformation which comprises incubating a sample of sperm or sperm nuclei (or a mixture thereof), in a dispersion extract and subsequently incubating said sample in a reprogramming extract. As discussed above, these extracts will preferably be derived from embryos or embryonic stem cells. Incubation in the dispersion extract preferably lasts for between 15 mins and an hour and a half, more preferably 30-60 mins and incubation in the reprogramming extract preferably lasts for ½-2½ hours.

In a further preferred embodiment ATP (0.5-1 mM) and creatine phosphate (5-10 mM) are added to the dispersion extract before and/or after the sperm (nuclei) are added for the first incubation step. The incubations will preferably be carried out at 25-35°C e.g. around 30°C.

The sperm (nuclei) are preferably contacted with Mg^{2+}, ATP and creatine phosphate as well as Extract B at the start of the second incubation.

GTP and glycogen are preferably added during the second incubation step.

Extracts A and B and methods for their preparation constitute further aspects of the invention. Preferred components of the extracts are discussed but they are most precisely described with reference to their methods of preparation.

By "dispersion" of the sperm nuclei is meant particularly decondensation of the nuclear chromatin as described, for example, in WO95/21860. Preferably

during this step sperm-specific protamines may be released and the initiation of the assembly of the new, larger, nuclear envelope begins. Chromatin dispersion and simultaneous initiation of nuclear envelope assembly is preferably achieved according to the method of the invention and this is particularly advantageous as it limits chromosomal breakdown. No prior art systems have achieved this for mammalian sperm.

The reaction of sperm nuclei to decondensation factors in the egg is likely to be a factor in determining fertility. Thus the dispersion and reprogramming extracts have utility in the field of reproduction such as fertility assessment, diagnosis of sub-fertility and infertility treatment.

The ability of sperm nuclei to disperse and reassemble a nuclear envelope are believed to be related to sperm parameters such as viability and motility which are indicative of their general health and functional capacity. The spermatozoa incubated in IVF media prior to ICSI progressively lost their ability to disperse and assemble nuclei (see Fig. 3). Thus the quality of sperm may be assessed on the basis of the response to incubation in the extracts of the present invention. Thus, in a further aspect the present invention provides a method of testing sperm, in particular for its ability to successfully fertilise an oocyte, which comprises incubating a sample of sperm, particularly sperm nuclei, in a dispersion and optionally a reprogramming extract and measuring the degree of chromatin decondensation and/or nuclear membrane assembly. Suitable techniques for monitoring chromatin decondensation and nuclear assembly, e.g. by visualisation using chromatin/chromosome dyes or fluorescent assays or immunoassays etc. are described in WO95/21860.

The reagents for use in such a diagnostic test may be packaged as a kit. Thus, in a still further aspect, the present invention provides a kit for testing sperm which comprises a dispersion and optionally a reprogramming extract as described above, optionally combined with one or more reagents for use in the detection of decondensed sperm chromatin and/or detection of a nuclear envelope.

Preferably, the dispersion results in enlargement of the sperm nuclei (5-15 times increase in size) within 15-30 min of incubation. Sperm nuclei resistant to dispersion fail to increase in size, and exhibit a distinctly impermeable sperm membrane, even after several hours of incubation (see Fig. 2).

During the second incubation step, the dispersed sperm nucleus undergoes "nuclear reprogramming", which refers to the process by which the sperm-specific chromosomal proteins (protamines) are released and new oocyte-specific (extract-specific) proteins are assimilated by the sperm chromatin to form a novel nuclear structure (also called chromatin remodelling). Additionally, the haploid sperm DNA is extensively and selectively modified by DNA methylases or demethylases present in the egg ooplasm (or extracts) to alter the pattern of methylation.

In a preferred embodiment magnesium ions, typically at concentrations in the region of 5-10 mM, are added to the reprogramming extract, optionally together with ATP and creatine phosphate, e.g. at concentrations of 0.5-1 mM and 5-10 mM respectively.

Also during the second incubation step, the sperm nucleus will preferably undergo complete or substantially complete reassembly of the nuclear envelope. Reassembly of the nuclear envelope begins

during the first incubation step. Preferably, the reprogramming of sperm nuclear chromatin and DNA repair takes place and glycogen (e.g. 0.5-3 mg/ml, such as 1 mg/ml) and GTP (preferably 0.5-1.0 mM) are added to facilitate fusion of membrane vesicles and formation of a discrete nuclear envelope. The small and tightly packed sperm nucleus has been transformed by dispersion and then reprogramming into a much larger unit which after reassembly of the lipid bi-layer nuclear envelope is morphologically much more similar to normal somatic cells or oocytes. Preferably the nuclear envelope will be at least 90% more preferably 95-100% complete after the second incubation step. This is determined by nuclear membrane-specific fluorescent staining or reacting the nuclei with antibodies against lamins.

In a particularly preferred embodiment the second incubation step is itself made up of two parts, a reprogramming of chromatin and DNA repair step which lasts for 15 to 60 mins and then a longer incubation period of 30 mins to 2 hours, typically 45 to 75 mins, after addition of GTP and glycogen during which the nuclear envelope forms. Although in alternative embodiments all components are added simultaneously and incubation continuous until the nuclear envelope is formed.

The dispersion extract (extract A) preferably contains lipids, typically lamins and membrane vesicles, which are utilised in the reassembly of the nuclear envelope. It is a particularly advantageous feature of the way in which the sperm nuclei are prepared in accordance with the method described above that fragments of the nuclear membrane remain and act as convenient nucleation points for the build up of a complete or substantially complete nuclear membrane after dispersion. Thus preferably, it is the sperm nuclei prepared by the permeabilisation

method described herein which are then subject to the above incubation/transformation techniques.

It is a particularly advantageous and novel feature of the present invention that the sperm dispersion can be controlled. Previous methods of dispersion led to a rapid breakdown of the chromosomes of the dispersed nucleus and thus the nucleus is not intact and available for fusion with other cells. In contrast, according to the methods of the present invention, the sperm nucleus is transformed to a normal nucleus with a nuclear envelope and is available intact for fusion with somatic cells or oocytes. The methods of the invention allow the size of the transformed sperm nucleus (with envelope) to be controlled to generate sperm nuclei for different purposes. The largest nuclei, diameter of approx. 45-40 micrometer are used for fusion with an oocyte, smaller nuclei having a diameter of approx. 20-25 micrometer fuse better with somatic cells and very small nuclei having a diameter of less than 20 micrometer are suitable for ICSI.

The eventual size of the transformed sperm nucleus can be controlled by the length of time of incubation, the longer the incubation period, the smaller the nucleus (see Fig. 6). This reduction of nuclear size on prolonged incubation is possibly due to the recondensation of the reprogrammed sperm chromatin. This transient condensation of the male pronuclear chromatin precedes fusion with female pronucleus during natural conception. We have observed that the reduction in nuclear size reaches a minimum (approx. 15-20 micrometer diameter) and remains unchanged even after several hours of additional incubation.

A further advantage is that the transformed sperm nuclei, once assembled, can be stored in liquid nitrogen

(-176°C) or at -70°C for up to 4-6 months.

Thus, according to a further aspect of the present invention is provided a transformed sperm nucleus whose chromatin has undergone decondensation and reprogramming and whose nuclear envelope has been reassembled to provide a nucleus which is at least 5 times, preferably at least 10-15 times larger than a normal untreated sperm nucleus.

Decondensation of chromatin, nuclear (chromatin) reprogramming and reassembly of the nuclear envelope are defined above. "Transformation" refers to the whole process by which a sperm nucleus undergoes extensive biochemical and morphological alterations so that it is capable of fusion with another cell and/or intracytoplasmic injection into an oocyte.

In a further aspect, the methods described herein to reprogramme sperm can also be used for reprogramming somatic cells. Nuclear reprogramming is the epigenetic transfonnation of a specific chromosomal organisation to a state which leads to localised or global activation and inactivation of genes. Global reprogramming is the characteristics of male pronuclear assembly and of the somatic cell nuclei transferred to oocytes observed in fertilisation and in animal cloning studies, respectively.

Nuclear reprogramming is the most critical step in animal cloning studies. In current techniques, the nucleus from the embryonic or adult cells is transferred to the enucleated oocytes. The developmental potential of the fully differentiated somatic cell nucleus is exclusively determined by the cytoplasmic factors of the oocyte and the competence of the chromosomes to undergo remodelling in the transferred nucleus.

However, the egg cytoplasm often fails to achieve successful reprogramming and this is why current animal cloning is very inefficient. It is therefore proposed to overcome these problems by reprogramming the differentiated somatic cell before it fuses with the enucleated oocyte. A particular advantage of reprogramming using the cell-free extracts described herein rather than relying on factors present in the oocyte cytoplasm is that while the amount of factors present in a single cell is limited, the extracts can be prepared to ensure that the cell is exposed to all necessary factors at adequate concentrations.

Thus somatic cells may be permeabilised as described herein, a preferred permeabilising agent being a mild detergent such as digitonin. An extract equivalent to the SNEB described herein is used but this typically contains a lower concentration of metal chelating agent e.g. <0.5 mM, preferably around 0.1 mM EDTA. The permeabilised somatic cells are then treated with Extracts A and B as described herein. The somatic cell nucleus is already fairly large (much larger than a sperm nucleus) and the methods may therefore result in little or no increase in overall size of the nucleus. However nuclear reprogramming takes place. Following reprogramming, the cells can conveniently be separated from the extracts by layering over (1-3 mL) of (1.1M) sucrose in SNEB and then centrifuged at 2.5K for 30 mins. The cells will settle at the bottom while extract proteins will remain at the top. These cells can then be used for fusion with enucleated oocytes according to known animal cloning techniques.

To assess the extent of remodelling/reprogramming, methylation of DNA and histones can be monitored by incorporating S-adenosyl methionine in the extract(s).

Two kinds of methylation changes would be expected: Global alteration in methylation of DNA (centromere and heterochromatin) and histones or localised effect on imprinted genes. Imprinted genes are marked by methylation in the gamete and can be erased only by germline transmission. Correct reprogramming in vitro would mean that methylation imprints passed on to the somatic cells by gametes (sperm or egg) would remain intact while methytation of other genes altered. These changes can be determined by standard molecular and biochemical methods.

Having prepared the sperm nuclei which are stable and transformed and have an intact nuclear envelope, there are many uses of the nuclei which have exciting therapeutic, diagnostic and scientific implications. Technologies have been developed to fuse two different normal (somatic) cells but these are not applicable to sperm-egg fusion because the human sperm are tiny, highly specialised and condensed cells. The transformed sperm nuclei of the invention are capable, through the morphological changes which they have undergone *in vitro*, to fuse with an oocyte or somatic cell.

Thus a preferred use of the transformed nuclei of the invention is in fusion with somatic cells or oocytes. In a further aspect, therefore, the present invention provides a method of fusion, e.g. chemical, biochemical or electrical fusion, electrical fusion (electrofusion) being preferred, between a transformed sperm nucleus and a somatic cell or oocyte. For the first time is provided a method of *in vitro* fusion between a sperm nucleus and the nucleus of another cell and thus a new method of *in vitro* fertilisation.

Electrofusion between the transformed sperm nucleus and an egg (oocyte) has many advantages over ICSI, the

conventional method for *in vitro* fertilisation for male factor infertility. It is simple, efficient (80-90%) and does not rely on operator ability; it is also much quicker many oocytes and sperm being fused in minutes. Mechanical damage to the oocyte by this method is negligible. Formation of the male pronucleus and electrical activation ensures subsequent formation of the female pronucleus which does not routinely happen in ICSI.

Standard electrofusion techniques may be used Collas, P., Fissore, R. and Robl, J.M., Anal. Biochem. 208: 1-9. 1993; Willadsen, S.M. Nature 320: 63-65, 1986; Bertsche, U. and Zimmermann, U. Radiat. Enviorn. Biophys. 27: 201-212, 1988; Lee, J.Y *et al.*, Nat. Genet. &: 29-33, 1994. Preferably, the sperm nuclei are pre-aligned on an electrode by a brief electrical pulse prior to mixing with eggs and electrofusion. Cell-tracking markers or specific genetic markers can be used to confirm a successful fusion as described in the Examples. A typical protocol comprises addition of a cell-tracking marker 15 mins before the end of the reprogramming/ reassembly incubation step. The nuclei are isolated by sucrose velocity gradient sedimentation and may be counted in a hemocytometer. The sperm nuclei can also be isolated individually by micromanipulation. The sperm nuclei and eggs are mixed at a ratio of 2:1 in a volume of approximately 60-80μl (Collas,P., Fissore, R. and Robl, J.M., Anal. Biochem. 208: 1-9. 1993).

Electrofusion can then be performed in an Electrocell Manipulator 200/Microslide (Genetronics, Inc. BTX Instrument Division, San Diego, California) with 1mm electrode gap. The embryo may then be cultured and optionally prepared for implantation so that it may proceed to term *in vivo*. The embryo may be cultured to blastocyst *in vivo* or *in vitro* and it may be desirable

to screen at the blastocyst stage for suitability for development to term, such methods are described, *inter alia*, in GB-A-2331751.

Mixing sperm and egg at a ratio of around 2:1 is particularly preferred in order to maximise the proportion of single sperm:egg fusion events, as opposed to sperm:sperm fusion, multiple sperm:egg fusion or egg:egg fusion. Using cuvettes with a capacity of 40-80μl and 1mm electrode gap, the sperm nuclei can be pre-aligned on the electrode by a brief electrical pulse prior to mixing with eggs and electrofusion. Single cells can be placed in BTX Microslide Cuvettes which can be fitted to a microscope with standard optics to provide the additional benefit of allowing visual alignment of a single egg and sperm nucleus between 0.5 mm stainless steel electrodes prior to electrical pulsing.

Methods of egg (oocyte) isolation from various animals are well-known in the art and any suitable technique for harvesting human or non-human oocytes may be used to provide oocytes for use in the fusion techniques of the present invention.

A further aspect of the present invention comprises an embryo prepared by *in vitro* fusion between a sperm nucleus and an oocyte. Typically the sperm nucleus will be a transformed cell nucleus prepared by one of the methods described herein. As discussed above, the present invention provides opportunities for IVF of great benefit to couples who are experiencing difficulties with conception. The invention is also of use in the preparation of non-human embryos, referred to herein as animal embryos which may be of particular use in agricultural contexts.

According to a further aspect of the invention is provided a method for preparing an animal, which method comprises preparing an animal embryo as described above, i.e. by *in vitro* fusion between a sperm nucleus and an oocyte and causing the animal to develop to term from said embryo and optionally breeding from the animal so formed. These latter stages may be performed as described in GB 2331751.

A further application of the present technology is in sperm-mediated gene transfer (transgenics). In this case the sperm nuclei can be pre-incubated with exogenous genes in a suitable vector prior to sperm dispersion.

Fusion of the sperm nuclei with oocytes has been discussed in some detail above. A further application of the present technology is in fusion with somatic cells in the diagnosis of sperm chromosome abnormalities. At present there are few techniques available for genetic analysis of sperm because of the dense packing of chromatin in the sperm heads.

In a further aspect, the present invention provides a method of testing for sperm chromosome abnormalities, which method comprises contacting a transformed sperm nucleus whose chromatin has undergone decondensation and reprogramming and whose nuclear envelope has been reassembled with a somatic cell and causing the two nuclei to undergo fusion (e.g. electrofusion) and subsequent cytogenetic and/or fluorescent in-situ hybridisation (FISH) analysis of the chromosomes to identify abnormalities. (Martini E. *et al.*, Hum. Reprod. 12: 2011-2018, 1997; Vegetti, W. et al., Hum. Reprod. 15: 351-365, 2000).

Suitable somatic cell types include Indian Female

Muntjac fibroblast cells or ES cells, cultured somatic cells generally being preferred. The chromosomes may conveniently be investigated by cytogenetic analysis using stains and paints.

Use of a transformed nucleus as described above in a method of testing for sperm chromosome abnormalities, particularly methods which involve electrofusion of said nucleus to another cell or cells constitutes a preferred aspect of the present invention. Diagnostic methods are conveniently performed using a kit and in a further aspect the present invention provides a kit for testing for sperm chromosome abnormalities which comprises Extract A and preferably Extract B as defined above, optionally together with a somatic cell capable of fusion with a sperm nucleus e.g. Indian female muntjac cells and optionally together with reagents for permeabilisation of sperm nuclei. Such agents, and an SNEB which may also be included are described above.

Reagents suitable for such isolation and transformation methods are described above. Means for chromosome analysis include culturing the fused cells in the presence of a selectable marker (drugs) and generating the metaphase chromosomes by temporary incubation in the presence of colcemid. The drug would allow the growth of only hybrid cells and colcemid would facilitate producing visible metaphase chromosomes.

As well as fusion between a transformed sperm nucleus as described herein and the nucleus of an oocyte or somatic cell, the transformed sperm nucleus may be injected into the cytoplasm of an egg in a manner analogous to ICSI. Thus in a further aspect the present invention provides a method of *in vitro* fertilisation which comprises microinjection of a transformed sperm nucleus as described herein (i.e. obtainable or obtained by the

processes described herein) into the cytoplasm of an oocyte. This technique has advantages over conventional ICSI techniques in that it avoids the need for oocyte induced reprogramming.

The invention will now be further described in the following non-limiting Examples with reference to the accompanying Figures in which:

Fig. 1a shows the experimental steps used to disperse sperm nuclei in Extract A which also provides membrane vesicles to initiate nuclear envelope assembly during the first hour of incubation. Addition of ATP and creatine phosphate (CP) at this step enhances sperm dispersion. Extract B, Mg^{2+}, additional ATP and CP allow chromatin remodelling and nuclear reprogramming to take place. GTP and glycogen aid fusion of the assembled vesicles to form a complete nuclear envelope.

Fig. 1b shows the various steps involved in sperm-egg fusion, sperm-mediated transgenics and methods of diagnosis of human sperm chromosome abnormalities.

Fig. 2 shows that Mg^{2+} added to the buffers during sperm nuclear isolation or added exogenously to the extracts, severely inhibits sperm nuclear dispersion. It is for this reason, that at the initial phase of incubation (Fig. 1a) Mg^{2+} is omitted. Phase contrast micrographs show the general morphology of the sperm under a light microscope, whereas, DAPI and HDCC highlight the DNA and nuclear envelope, respectively, under fluorescent microscope.

Fig. 3 demonstrates that continued incubation of gradient purified human spermatozoa in IVF medium (Medicult) inhibits sperm dispersion. The staining of the spermatozoa with lipophilic fluorescent stain HDCC

suggests that increased stability of the sperm nuclear membrane on prolonged incubation could be responsible for the resistance of the sperm to dispersion. This figure also shows that addition of ATP and CP during initial phase of incubation in Extract A (Fig. 1a) enhanced sperm dispersion.

Fig. 4 shows mouse sperm nuclear dispersion, chromatin remodelling and very early stage of re-assembly of the nuclear envelope. The steps used are shown in Fig. 1a. Very early phase of sperm dispersion is shown in D, E and F (compare with controls A-C). Membrane vesicles are already associated with the dispersed sperm (F) in the first hour of incubation. When extract B, Mg^{2+} and CP were added, the chromosomes of the dispersed nuclei underwent reprogramming and the nuclear envelope became more defined (G to L).

Fig. 5 shows continued incubation of remodelled nuclei in the presence of GTP and glycogen facilitates a) fusion of the vesicles to produce a distinct nuclear envelope and b) reduction in the size of the nucleus. The envelope assembled around remodelled nuclei (Fig. 4) became distinct (A and B) and the nuclei became smaller on further incubation (compare a & b with c and d in A). These nuclei can be stored at -70°C without changing the morphology on thawing.

Fig. 6 demonstrates transformation of human sperm nuclei at various stages as shown for mouse sperm in Figs. 4 & 5. The sperm nuclei dispersed within 1 hour of incubation (A-C), gradually became more dense and smaller on further incubation (D to L). Occasionally, some sperm nuclei (but less than 1%) failed to undergo transformation even after hours of incubation as shown in J, K and L.

Fig. 7 comprises photographs showing the relative size and morphology of human sperm, remodelled (i.e. reprogrammed) sperm and muntjuc cells.

Fig. 8 comprises photographs showing the chromosome composition of muntjac and hybrid cells which were selected for drug resistance after fusion with human sperm nuclei.

Fig. 9 comprises photos of hybrid cells and human lymphocytes which indicate the presence of human chromosomes in the muntjac hybrid cells.

EXAMPLES

Reagents

All standard chemicals (Dithiothreotol, DTT, Potassium chloride, Potassium acetate; Magnesium chloride and Magnesium acetate, Sodium chloride, Sucrose, Glycerol, ethanol etc) were molecular biology grade from BDH. Nucleotides (ATP and GTP), Percoll (Molecular biology grade) were from Amersham-Pharmacia. Micrococcal nuclease (MiNase) and Deoxyribonuclease I (DNase I), Restriction enzymes, glycogen and proteinase K, protease inhibitor cocktails were from Promega/Amersham-Pharmacia or Boehringer & Manheim. Lysolecithin, Digitonin, Dimethylsulphoxide, Creatine Phosphate, Propidium Iodide, DAPI, Vectachield and reagents for recombinant DNA work were from Sigma Chemicals. HDCC, a lipophilic fluorescent membrane stain was from Calbiochem. Cell culture media, antibiotics, cell freezing media, trypsin-EDTA, colcemid were from Life Technologies. Dialysis tubings (Mr cut of 3, 500) were from Spectraphor.

Preparation of Mouse embryo and embryonic stem (ES) cell-free extracts: Extracts A and B

Developing mouse embryos were obtained from female mice (C57 BL/6) at 11th or 12th day of pregnancy. The placenta and other extra embryonic tissues were surgically removed under low-magnification microscope and the embryos were transferred to ice-cold PBS in a sterile beaker held on ice. Henceforth, all the operations were carried out on ice or at 4°C.

The embryos were dissected into small fragments and were extensively washed with ice-cold PBS The tissue sections were transferred to a loose-fitting glass homogenizer and were further reduced to smaller sizes (6-8 strokes). The material was spun at 1200 rpm for 5 min. and the supernatant was discarded. The embryonic tissues were resuspended in 20 mM HEPES-NaOH, pH 8.0, 10 mins KCl, 0.5 mM DTT and 1X protease inhibitors cocktail and held on ice for 10 mins.

The material was transferred to a tight-fitting Teflon-coated glass homogenizer and homogenized on ice with 8-10 strokes. The homogenate was transferred to sterile plastic tubes and spun at 2,000 rpm for 15 mins. The supernatant (S) was carefully removed to another fresh tube and the pellet (P) containing the rest of the tissues was held on ice. The KCl concentration in S-fraction was adjusted to 100 mM, centrifuged at 2,500 rpm prior to dialysis and designated as Extract A. The P-fraction was resuspended in a buffer containing 20 mM HEPES, pH 8.0, 0.2 mM EDTA, 0.5 mM DTT, 5% glycerol and 1X protease inhibitors cocktail prior to adding 0.6 M NaCl in small drops. The suspension was gently mixed for 15 min and spun at 13,000 rpm for 30 min.

The supernatant was carefully removed (without

disturbing the pellet) and additional glycerol was added to achieve final concentration to 20%. This material after dialysis was called Extract B. The dialysis was carried out in 400-500 volumes of a buffer (20 mM HEPES-NaOH, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 20% glycerol) for 6 h with one change. The dialysis bag was from Spectraphor with a molecular weight cut off of 3,500. Dialysis tubes with higher molecular weight cut off increases the precipitation of proteins resulting in poorer quality extracts. Following dialysis, the extracts were further centrifuged at 4,300 rpm for 15 mm and the supernatant was aliquoted, snap frozen in liquid Nitrogen and stored for at least 6 months at -70°C without losing activities.

Mouse embryonic stem cells (EFC-1, passage 16, obtained from Western General Hospital, Edinburgh were plated on mitomycin-treated feeder layers (STO) in 90-mm pre-gelatinised plastic dishes and cells were routinely maintained undifferentiated by growing in the presence of LIF (leukocyte inhibitory factor). In order to obtain large number of cells, the cells were subcultured by plating without feeder layer (STO) for 2 h and subsequently transferring the media containing unattached ES cells to fresh gelatin-coated dishes. This procedure removes the residual feeder cells. The ES cell colonies were detached from the plates using rubber-policeman in cold PBS and were used to prepare cell-free extracts exactly as described for mouse embryos.

Generally, none of our buffers contain Mg^{2+} or other divalent cations. Conventionally, the cell-free extracts reported for studying transcription, DNA replication or repair *in vitro* contain varying concentrations of Mg^{2+}. As we shall discuss below, Mg^{2+} is required for nuclear remodelling and certain steps of

nuclear envelope assembly in our system. Therefore, we add magnesium exogenously at a specific time point during incubation. It must be emphasized however that cationic interference is only prominent at the initial phase of incubation during sperm dispersion. Once the sperm dispersion is complete, Mg^{2+} has no effect. On the contrary, this divalent cation helps the rest of the processes during incubation. Based on this logic, we have also prepared Extract B (but not extract A) in buffers containing 5 mM Mg^{+2} and they work well.

Unlike reported cell-free extracts, we find that high speed centrifugation (60-70, 000 rpm/>200,000g in an ultracentrifuge) of extracts (more specifically Extract A) significantly reduced the sperm dispersion activity and dramatically affected the nuclear envelope assembly.

Preparation of mouse and human sperm nuclei

The whole epididymis and vas deferens were recovered from 42-48 sexually mature (50-70 day old) C57BL/6 mice in a Petridish containing cold phosphate buffered saline without Ca^{2+} and Mg^{2+}. The epididymis were opened by sharp incisions with a razor blade and the mature spermatozoa were allowed to swim out by gently agitating the dish for 10-15 mm at room temperature. The spermatozoa released in the buffer were filtered through 8 layers of gauze to remove the tissue debris. The filtrate was spun at 1200 rpm for 15 mm at 4°C.

The pellet was washed 2-3 times in sperm nuclear extraction buffer (SNEB: 20 mM HEPES-NaOH, pH 7.0, 150 mM KCl, 250 mM sucrose, 10 mM EDTA and 1X protease inhibitors cocktail (antipain, aproteinin, leupeptin, chymostain and APMSF) and was resuspended in the same buffer. Mature spermatozoa were separated from less differentiated spermatocytes by sedimenting through 40%

percoll gradients equilibrated with SNEB. The gradient purified spermatozoa were resuspended in SNEB containing 1-α-phosphotidyl choline (lysolecithin, Sigma), 10 mM DTT and sonicated for 30 seconds (10 seconds each time). It has also been found that the lysolecithin concentration could be reduced 10-fold by adding digitonin (100-200 μg/ml) in the SNEB during permeabilisation. The spermatozoa were permeabilised on ice for 15 min (regular agitation) and the reaction was stopped by adding 3 volumes of SNEB containing 3 mg/ml bovine serum albumin (BSA).

In order to separate tails from the sperm head, the pellet resuspended in a small volume was layered over 1.1M sucrose cushion and was sedimented by centrifugation at 4500 rpm for 30 min at 4°C. The nuclei recovered following centrifugation were washed with SNEB containing 10 mM DTT. The number of nuclei/ml was determined by counting several dilutions in a haemocytometer and the preparation was stored in SNEB containing 50% glycerol at -70°C.

Preparation of human sperm nuclei

Human semen samples were obtained from healthy donors at the St. George's Assisted Conception Unit (ACU). The semen was liquefied at 37°C for 30 min prior to separation of the motile mature spermatozoa by discontinuous silica-based density gradients (recommended for human sperm preparation). The gradient purified spermatozoa were washed in PBS and the nuclei were prepared as described above with the following exceptions: The brief sonication step, final sedimentation by layering the permeabilised nuclei over 1.1 M sucrose cushion were omitted and EDTA concentration in SNEB was reduced to 3-5 mM and 0.25 mM (each) of spermine and spermidine added to avoid

clumping of the nuclei (Spermine and spermidine were not used for mouse sperm nuclear preparation). The brief sonication step was avoided because unlike mouse sperm, the tails of the human sperm usually dissociate from the head during permeabilisation and/or incubation in extracts. After permeabilisation and transformation typically 90-95% of human sperm tails are lost.

Sperm dispersion, nuclear reprogramming and re-assembly of nuclear envelope in cell-free extracts (embyonic or embryonic stem, ES cells).

A general outline of the procedures are presented in Fig. 1a. 2-3 x 10^5 lysolecthin plus digitonin permeabilised sperm nuclei (human or mouse) were resupended in 50 μl buffer (20 mM HEPES-NaOH, pH 8.0, 150 mM KCl) containing 0.5 mM DTT, 0.5 mM ATP, 5-10 mM creatine phosphate, 1X protease inhibitors cocktail and 10-20 μl Extract A (70-100 μg of total protein) and the materials in the tube were gently mixed on ice prior to incubation at 30°C. After 30 min of incubation, a 2 μl aliquot was mixed with fluorescent dyes reconstituted in above buffer (DAPI plus HDCC or PI alone) on a microscope slide and was examined in an epifluorescent microscope (Nikon, Eclipse, E 400) connected to a system for image processing and analysis (LUCIA, version 4.21). When >90-95% of the nuclei dispersed (takes between 30-60 min), the tube was transferred to room temperature and 15-20 μl Extract B (225-300 μg of total protein) was added, and the ATP, CP and Mg^{2+} concentrations were adjusted to 0.5 mM, 5-10 mM and 5-6 mM respectively, in a 100 μl total volume. Following 30 min of further incubation, GTP and Glycogen were added to 0.5-1.0 mM and 1-2 mg/ml respectively. The envelope assembly was examined and the images were saved. The nuclei with re-assembled nuclear envelope can be stored at -70°C. The nuclear reprogramming was examined by molecular methods.

Isolation and culture of mouse oocytes

For immature oocytes, B6CBF1 (21-24 days) or MF1 female mice (21 days) are injected with 7.5 IU eCG. The ovaries are removed 48-52h post-injection into M2 medium (Sigma) containing 10% fetal bovine serum (ICN Flow) and 0.25 mM dibutyryl cyclic AMP (db CAMP, Sigma). The oocytes released by puncturing large antral follicles with a 25G hypodermic needle are denuded by repeated pipetting *in* the presence of a low concentration of hyaluronidase (Sigma). To obtain mature oocytes, the animals are injected with eCG 48-52 h prior to second injection with 5IU of hCG. The cumulus masses recovered by squeezing the oviduct 13.5-16 h post hCG in M2 medium containing fetal bovine serum and the mature oocytes denuded by hyaluronidase treatment are maintained in M2 media prior to fusion.

Embryonic stem cell and Indian female muntjac fibroblast culture:

These may be prepared by standard methods as described, for example, in Sen, P and Sharma, T. Cytogenet. Cell Genet. 39: 145-149, 1985; Lee, J.Y. *et al.*, Nat. Genet. 7: 29-33, 1994.

Electrofusion and culture of the embryo:

A cell-tracking fluorescent probe chloromethyl derivative of fluorescin diacetate (CMFDA), which forms an adduct with intracellular thiols and is stable for several generations, is added during incubation of sperm nuclei in extracts (Fig. 1). The cell-tracking marker is incorporated into sperm during nuclear remodelling in the cell-free systems. The marker is colourless and nonfluorescent until cytosolic esterases cleave off the acetate, releasing a brightly green fluorescent product

The sperm nucleus fused to an egg activates the fluorophor to produce a green fluorescence (at visible wavelength) of viable embryos resulting from sperm-egg fusion. Alternatively, a specific genetic marker (e.g. neo-lacZ vector) is pre-incubated with sperm nuclei prior to nuclear transformation in cell-free extracts and expression of these proteins (drug resistance and staining) in the embryo or fused cells confirms successful fusion and transgenesis.

The efficiency of fusion technology has improved dramatically (80-90%) in the past 2-3 years. This is primarily due to improved alignment of cells, better design of fusion chambers and cell-tracker monitoring systems. Microslide cuvettes (BTX) with a capacity of 40-80 μl and 1 mm electrode gap may be used. In these cuvettes the sperm nuclei could be pre-aligned on the electrode by a brief electrical pulse (AC, pearl string) prior to mixing with eggs and electrofusion (DC).

Suitable electrofusion techniques are known in the art and described, for example, in Takahashi, M. *et al.*, Leukemia Research 15: 507-513, 1991; Nishi, T. *et al.*, Cancer Research 56: 1050-1055, 1996; Meng, J. *et al.*, Biol. Reprod. 57: 454-459, 1997; Saunders, J. Guide to electroporation and Electrofusion, 227-247, 1991; BTX Protocols PR0182, PR0336, PR0342, 1998; Miramatsu *et al.*, Int. J. Mol. Med. 1: 55-62, 1998.

Embryo culture and uterine transfer

Standard methods are suitable and described, for example, in Gordon, J.W. Metods Enzymol. 225: 747-771, 1993; Kimura, Y. and Yanagimachi, R. Biol. Reprod. 52: 709-720, 1995; Lacham-Kaplan, O. and Trounson, A. Hum. Reprod. 10: 2642-2649, 1995; Wakayama, T. et al., Nature 349: 369-374, 1998; Perry, A.C. et al., Science 284:

180-183, 1999.

Sperm-somatic cell fusion

The Human sperm nuclei were reprogrammed into somatic-type nuclei in mouse embryonic cell-free extracts as described herein.

The relative size and morphology of the *in vitro* reprogrammed human sperm nuclei and muntjac cells are shown in Fig. 7. Muntjac cells were grown to semiconfluence (12-16h) in 90 mm Petri dishes. The cells were washed three times with serum-free media prior to treatment with phytohaemaglutinin (100-200 μg/ml) resuspended in the same media. After 1h incubation at 37°C, the excess phytohaemoaglutinin was removed and sperm nuclei remodelled in the presence of *Neo*^{r} plasmid marker were added. Following incubation for 2h, the excess unattached sperm nuclei were removed and fusion was carried out for 1 mm in the presence of 50% poleethyleneglycol (PEG). The PEG was removed by washing the plates at least 4-5 times with Ca^{+2}/Mg^{+2} free PBS. The fused cells were maintained in complete media for 48h. The drug-resistant clones were selected for 3-4 wks in the presence of 500-1000 μg/ml of G418. The drug resistant clones were picked individually and were expanded in complete media without drug by sequential transfer to multi-well plates. The cells were treated with 0.5 μg/ml colcemide for 6h to examine the metaphase chromosomes of the fused cells (Fig. 8). The presence of human chromosome was determined by fluorescent in situ hybridisation (FISH) using human pan-centromere probe. As a control, the human lumphocyte metaphase chromosomes were hybridised under identical conditions (Fig. 9).

Claims

1. A method of sperm permeabilisation comprising:

(a) washing a sample containing sperm in a sperm nuclear extraction buffer (SNEB) which includes one or more metal chelating agents and which is substantially free of non-ionic detergents and divalent cations;

(b) contacting the sperm with one or more permeabilising agents; and

(c) optionally separating the sperm tail from the sperm head.

2. A method as claimed in claim 1 wherein the metal chelating agent is selected from the group comprising EDTA, EGTA and Bathophenanthroline sulphonate.

3. A method as claimed in claim 1 or claim 2 wherein the permeabilising agent is lysolecithin or digitonin.

4. A method as claimed in any preceding claim wherein the SNEB contains one or more protease inhibitors.

5. A method as claimed in any preceding claim wherein after contact with a permeabilising agent, parts of the nuclear membrane remain intact.

6. A method as claimed in any preceding claim wherein the SNEB contains spermine and/or spermidine.

7. A method of sperm transformation which method comprises incubating a sample of sperm or sperm nuclei in one or more cell-free extracts.

8. A method as claimed in claim 7 wherein the cell-free extracts are derived from embryos or embryonic stem cells.

9. A method as claimed in claim 7 or claim 8 wherein the sperm or sperm nucleus is permeabilised prior to incubation in the cell-free extract.

10. A method as claimed in any one of claims 7 to 9 wherein said sperm or sperm nuclei are incubated in a cell-free dispersion extract and in a cell-free reprogramming extract.

11. A method as claimed in claim 10 wherein said dispersion extract contains the necessary components for reassembly of a nuclear envelope.

12. A method as claimed in claim 10 or 11 wherein the dispersion extract comprises a supernatant generated by centrifugation of a cell sample.

13. A method as claimed in claim 12 wherein said cell sample is a sample of embryos or embryonic stem cells.

14. A method as claimed in claim 12 or claim 13 wherein the reprogramming extract comprises a supernatant generated following resuspension and centrifugation of the pellet formed on generation of the supernatant used in the dispersion extract.

15. A method as claimed in claim 14 wherein the reprogramming extract contains magnesium ions.

16. A method as claimed in claim 14 or 15 wherein the supernatant used in the reprogramming extract undergoes a further centrifugation step.

17. A method as claimed in any one of claims 10 to 16 wherein the sperm or sperm nuclei are incubated in said dispersion extract for between 15 and 90 mins and in said reprogramming extract for between 30 and 150 mins.

18. A method as claimed in any one of claims 10 to 16 wherein said dispersion extract contains ATP and creatine phosphate.

19. A method of preparing a sperm dispersion extract from a cell sample which comprises:

(a) suspending the sample in a buffer which is substantially free of divalent cations;

(b) centrifuging the sample at 1,000-5,000 rpm for 5 to 25 minutes; and

(c) collecting the supernatant product of step (b).

20. A method of preparing a sperm reprogramming extract which comprises:

(a) suspending the pellet product of step (b) of claim 19 in a buffer which optionally contains magnesium ions;

(b) centrifuging the suspended pellet of step (a) at 8,000-20,000 rpm for 15-45 mins; and

(c) collecting the supernatant product of step (b).

21. A method as claimed in claim 19 or 20 wherein the cell sample is an embryo or embryonic stem cell sample.

22. A method as claimed in claim 17 wherein the incubation in said reprogramming extract is in two

parts, a first part which lasts for 15 to 60 minutes and a second part which lasts for 30 to 120 minutes and takes place in the presence of GTP and glycogen.

23. A method of testing a sample of sperm or sperm nuclei which comprises:

(a) incubating the sample in a cell-free dispersion extract as defined in any one of claims 11 to 21;

(b) optionally further incubating the sample in cell-free reprogramming extract as defined in any one of claims 13 to 21; and

(c) measuring the degree of chromatin decondensation and/or nuclear membrane assembly.

24. A kit for use in a method as claimed in claim 23 which comprises:

(a) a cell-free dispersion extract as defined in any one of claims 11 to 21;

(b) optionally a reprogramming extract as defined in any one of claims 13 to 21; and

(c) optionally one or more reagents for the detection of decondensed sperm chromatin and/or detection of a nuclear envelope.

25. A method or kit as claimed in any one of claims 10 to 24 wherein said dispersion extract contains lipids.

26. A method or kit as claimed in claim 25 wherein said dispersion extract contains lamins and/or membrane vesicles.

27. A transformed sperm nucleus obtainable by the method of any one of claims 7 to 18.

28. A transformed sperm nucleus whose chromatin has undergone decondensation and reprogramming and whose nuclear envelope has been reassembled to provide a nucleus which has a diameter of 15 to 50 micrometers.

29. A method of *in vitro* fusion between a transformed sperm nucleus and a somatic cell or oocyte.

30. A method as claimed in claim 29 wherein the transformed sperm nucleus is as claimed in claim 27 or claim 28.

31. A method of electrofusion between a transformed sperm nucleus as claimed in claim 27 or claim 28 and an oocyte.

32. A method as claimed in claim 31 wherein the sperm nuclei and oocytes are mixed at a ratio of 1:1 to 3:1.

33. A method of testing for sperm chromosome abnormalities, which method comprises contacting a transformed sperm nucleus as claimed in claim 27 or claim 28 with a somatic cell and causing the two nuclei to undergo fusion and subsequent analysis of the chromosomes.

34. A method as claimed in claim 33 wherein the chromosomes are analysed by cytogenic or fluorescent in-situ hybridisation analysis.

35. A kit for use in a method as claimed in claim 33 or 34 which comprises:

(a) a cell-free dispersion extract as defined in any one of claims 11 to 21;

(b) optionally a reprogramming extract as defined in any one of claims 13 to 21; and

(c) optionally a sample of somatic cells capable of fusion with a sperm nucleus.

36. A method of *in vitro* fertilisation which comprises microinjection of a transfused sperm nucleus as claimed in claim 27 or claim 28 into the cytoplasm of an oocyte.

37. A method of reprogramming a somatic cell which method comprises incubating said somatic cell in one or more cell free extracts.

38. A method as claimed in claim 37 wherein the cell-free extracts are derived from embryos or embryonic stem cells.

39. A method as claimed in claim 37 or claim 38 wherein said somatic cell is incubated in a cell-free dispersion extract and in a cell-free reprogramming extract as defined in any one of claims 11 to 18.

40. An animal embryo prepared by transferring the reprogrammed nucleus of a diploid donor cell i nto a suitable recipient cell.

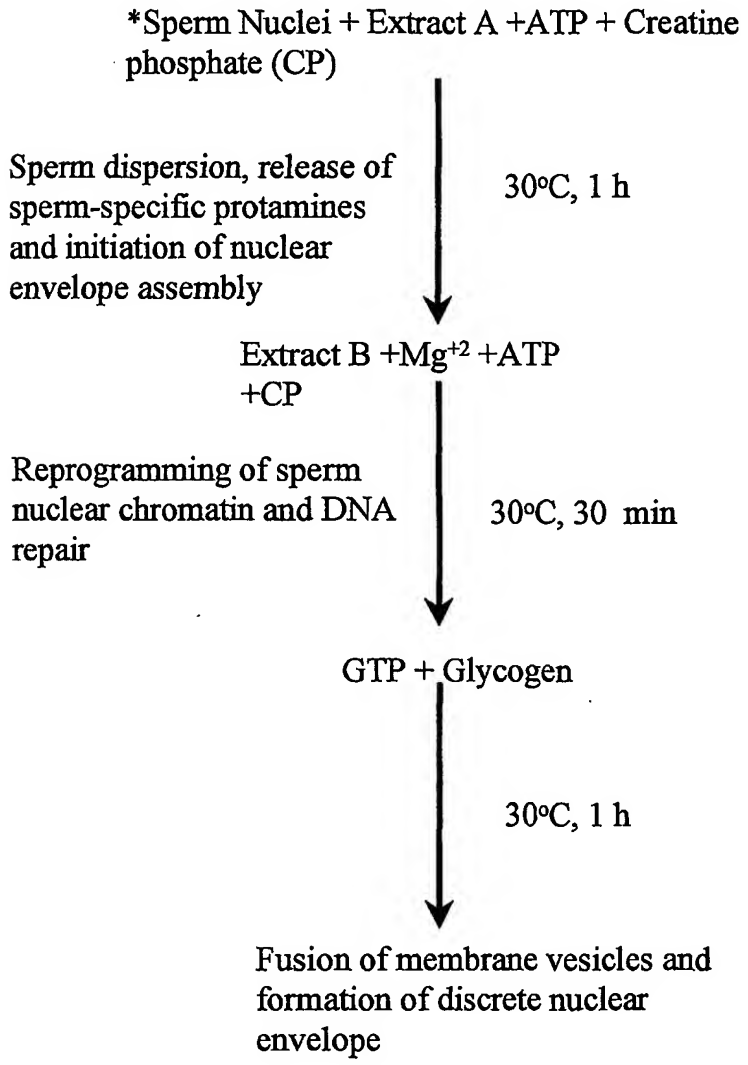
41. An embryo as claimed in claim 40 wherein the recipient cell is an oocyte.

42. An embryo as claimed in claim 40 or 41 wherein the diploid donor cell has been reprogrammed using a method as claimed in any one of claims 37 to 39.

43. A recipient cell into which the reprogrammed nucleus of a diploid cell has been introduced.

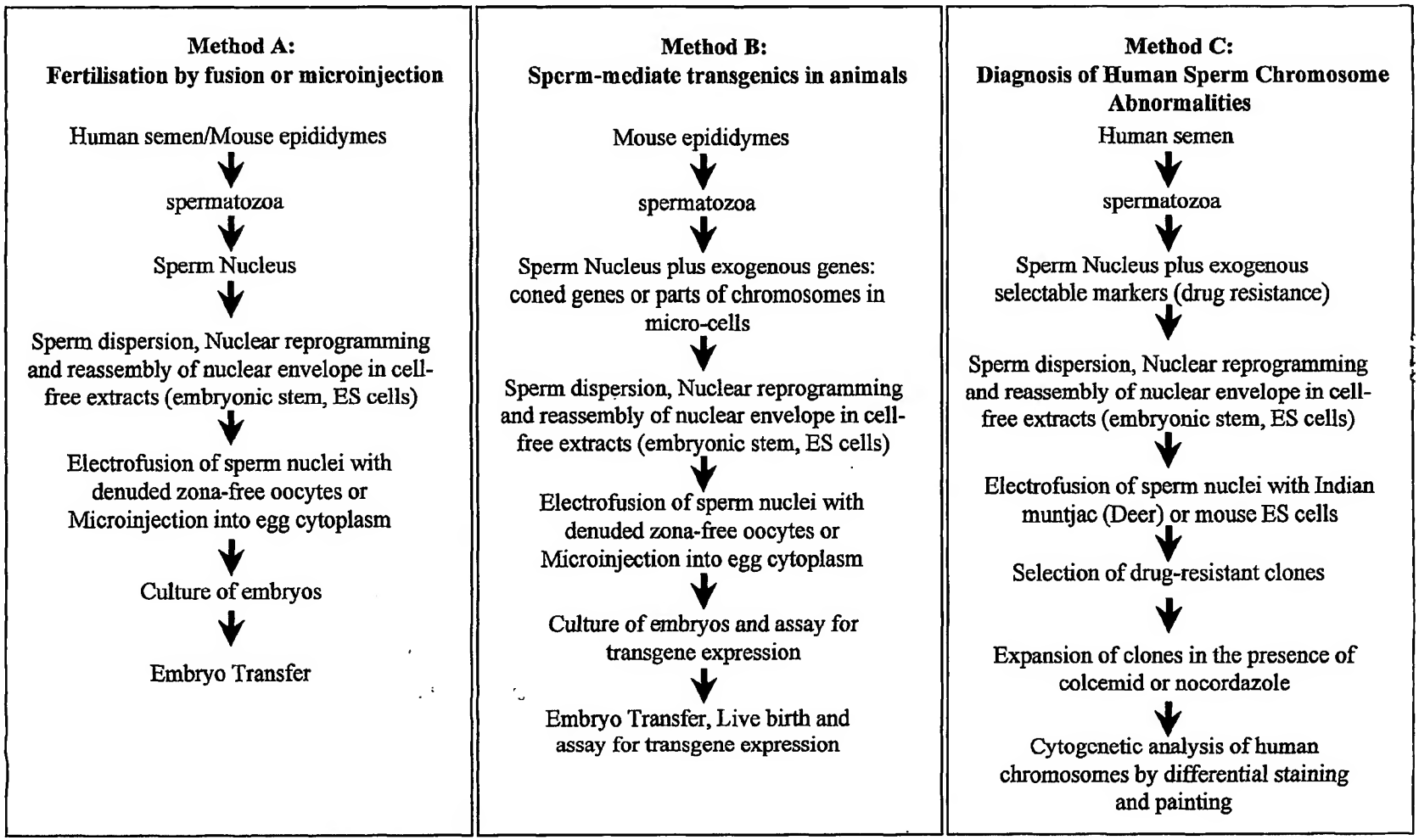
44. A cell as claimed in claim 43 wherein the recipient cell is an oocyte.

Fig. 1 a Sperm dispersion, Nuclear reprogramming and Re-assembly of nuclear envelope in cell-free extracts (developing mouse embryos/embryonic Stem, ES cells)



* Where sperm-mediated gene transfer (transgenics) is intended, the sperm nuclei are pre-incubated with a vector containing drug-resistant gene (neomycin, G418) and a colour selection marker gene (lac Z).

Fig. 1b A schematic presentation of the methods of sperm-egg fusion, sperm-mediated transgenics and and diagnosis of human sperm chromosome abnormalities



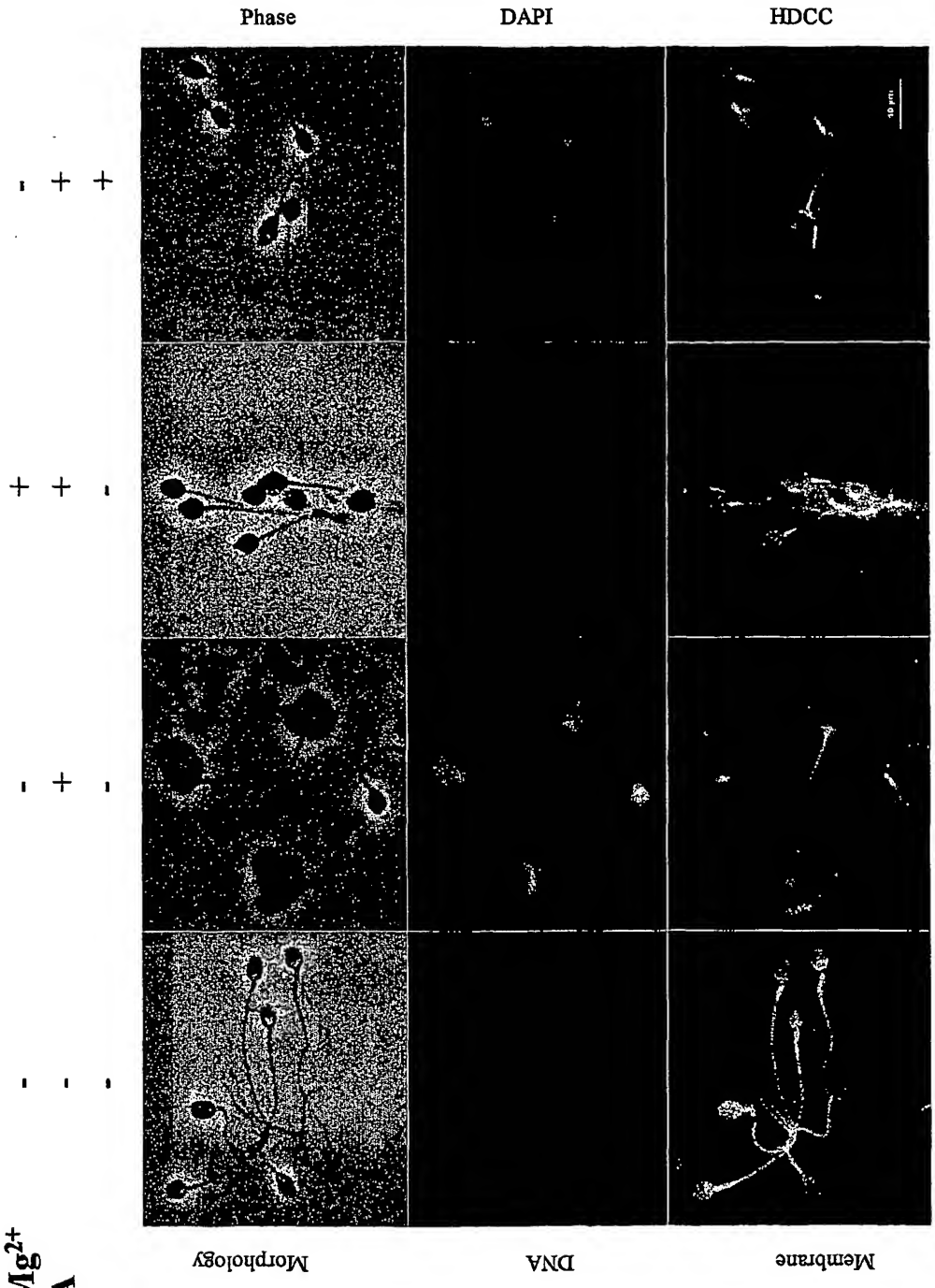


Fig.2 Inhibition of human sperm dispersion by Mg^{2+}.

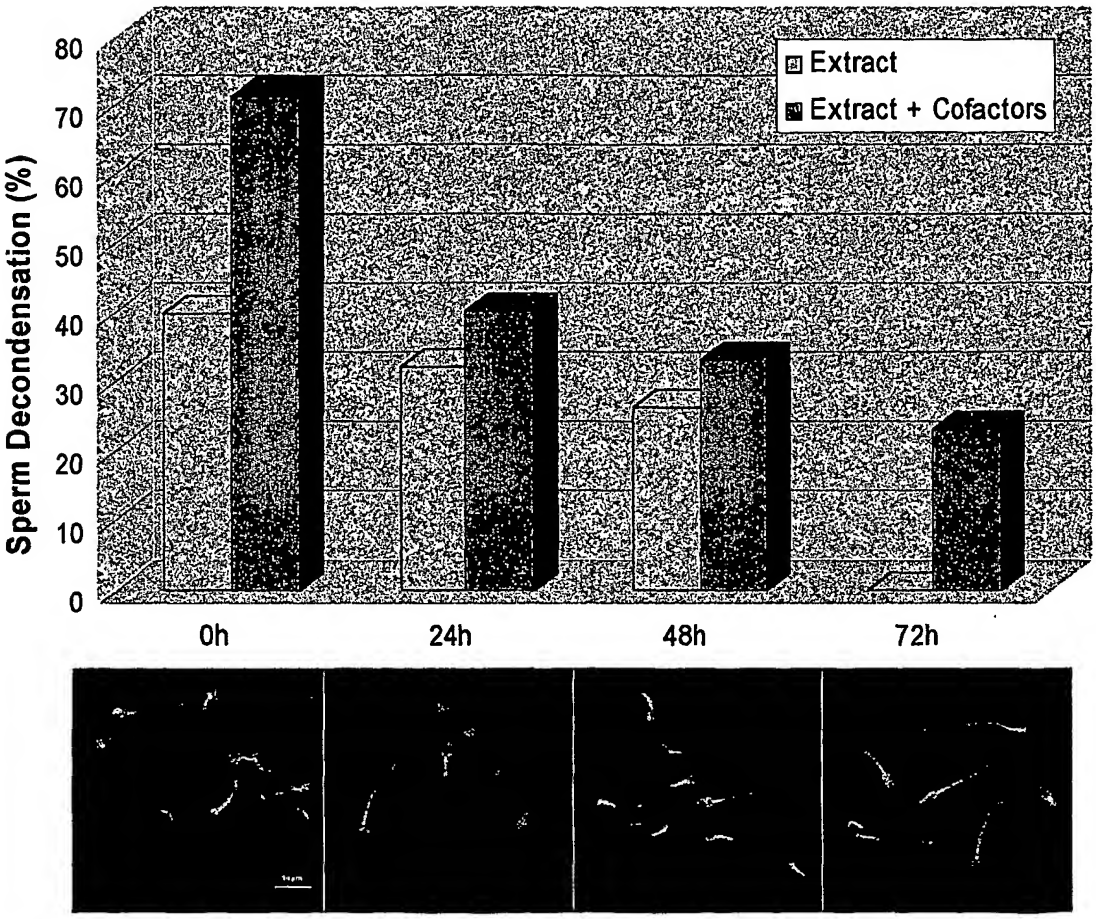


Fig.3 Prolonged incubation of human sperm in IVF medium (Medicult, UK) is detrimental to nuclear dispersion

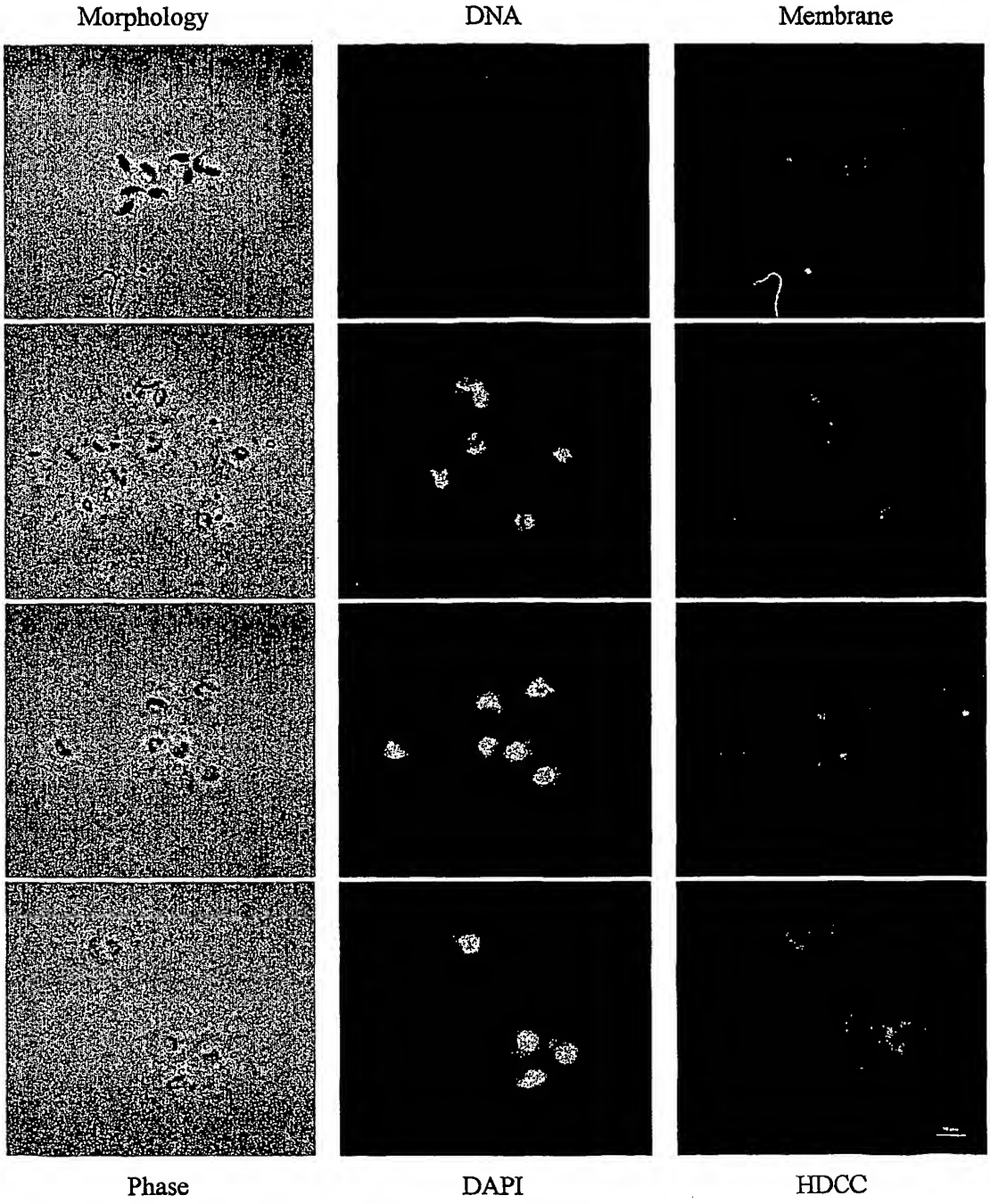


Fig.4 Mouse sperm dispersion, genomic reprogramming and early stages of re-assembly of the nuclear envelope

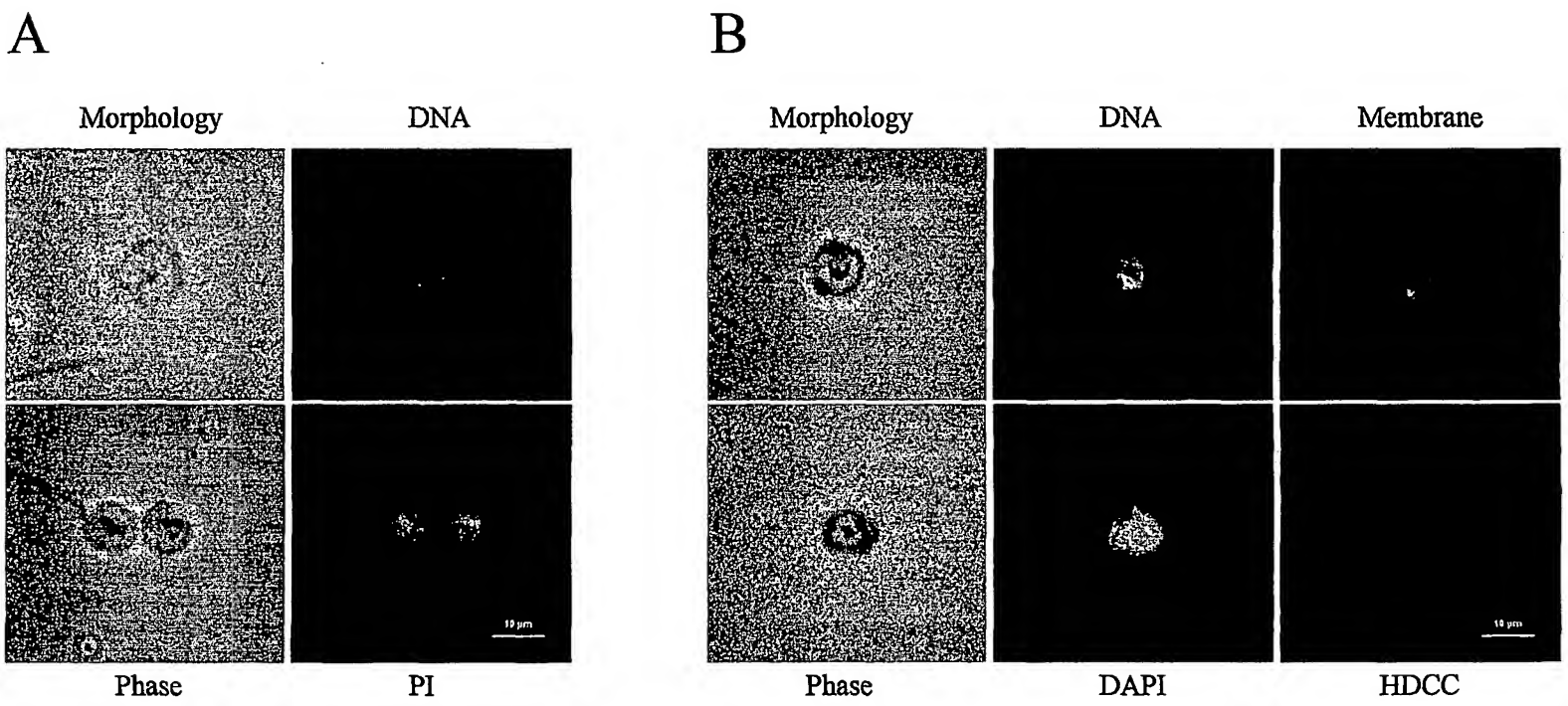


Fig. 5 Mouse sperm nuclear size can be controlled following assembly.
A & B are results from two independent experiments

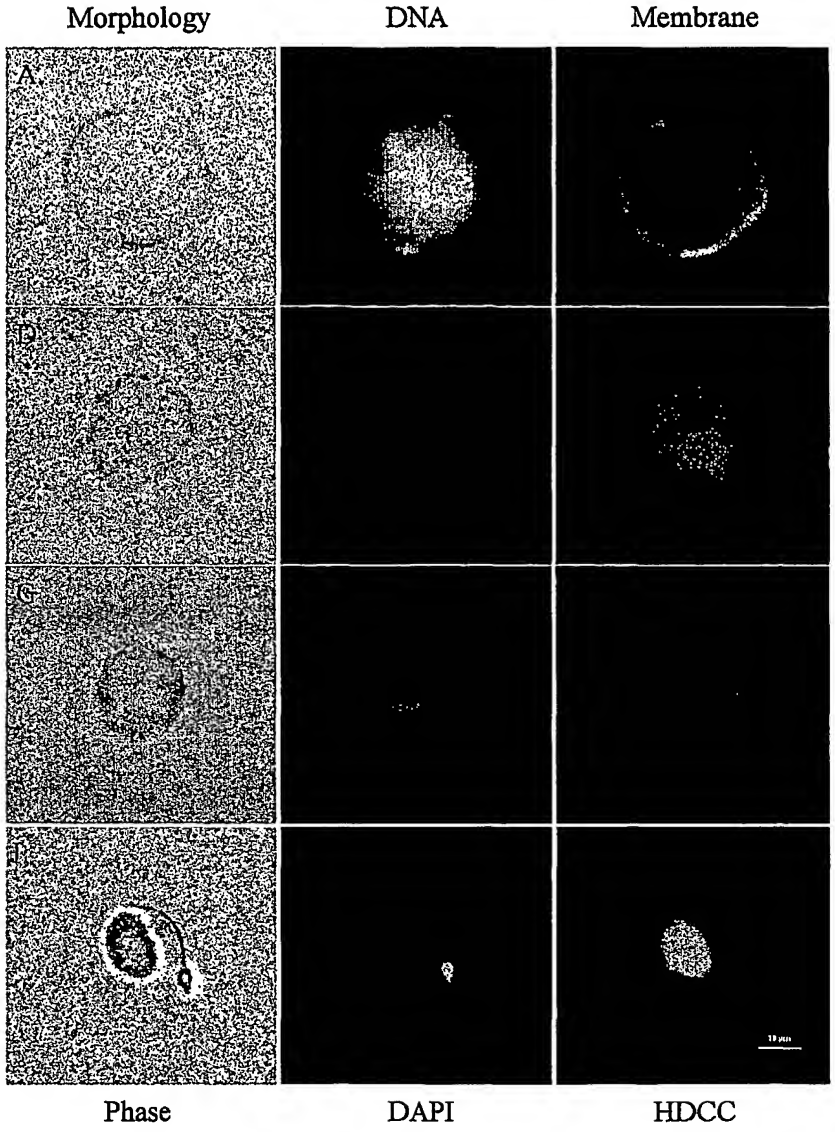


Fig. 6 Human sperm dispersion, genomic reprogramming and reassembly of nuclear envelope.

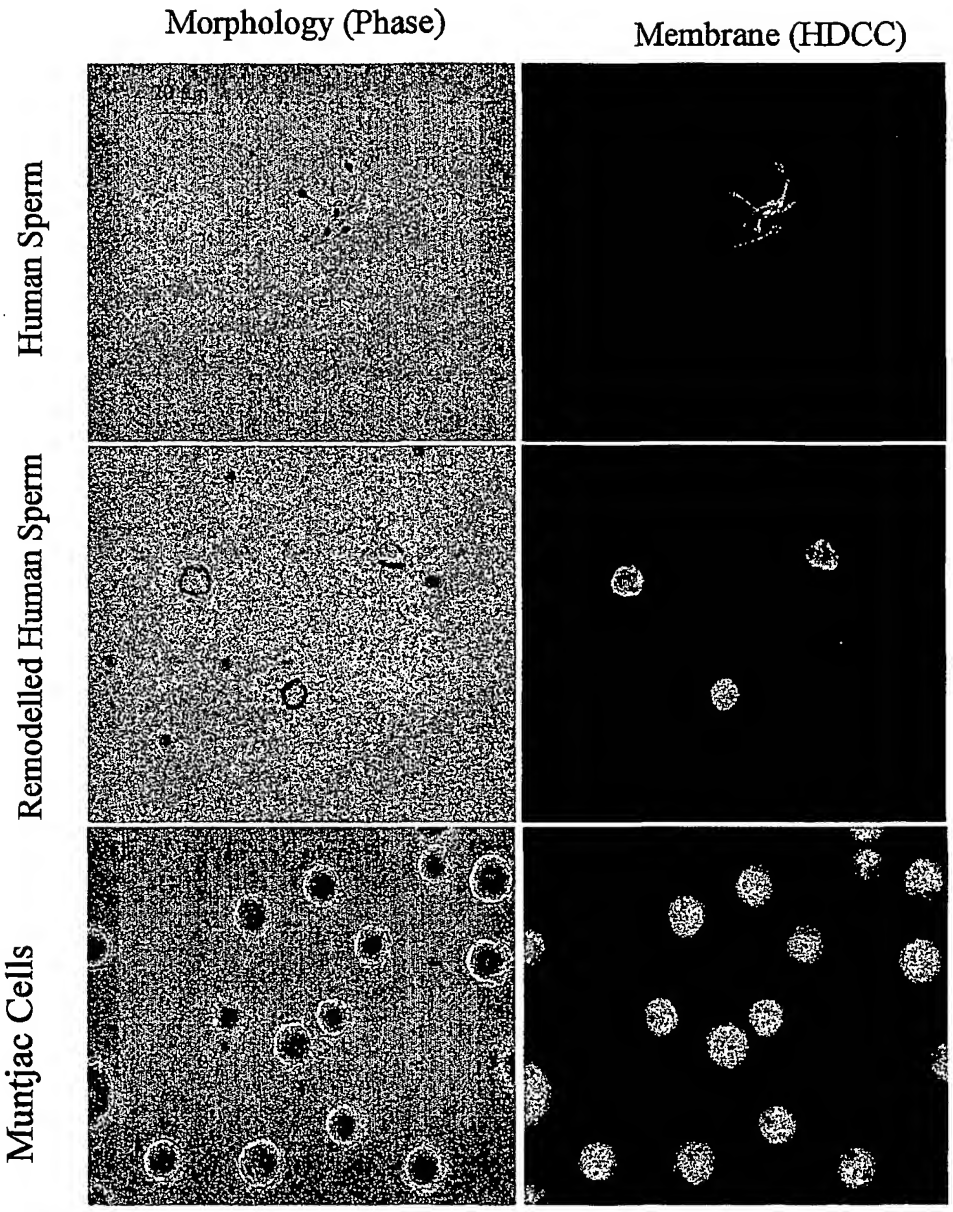
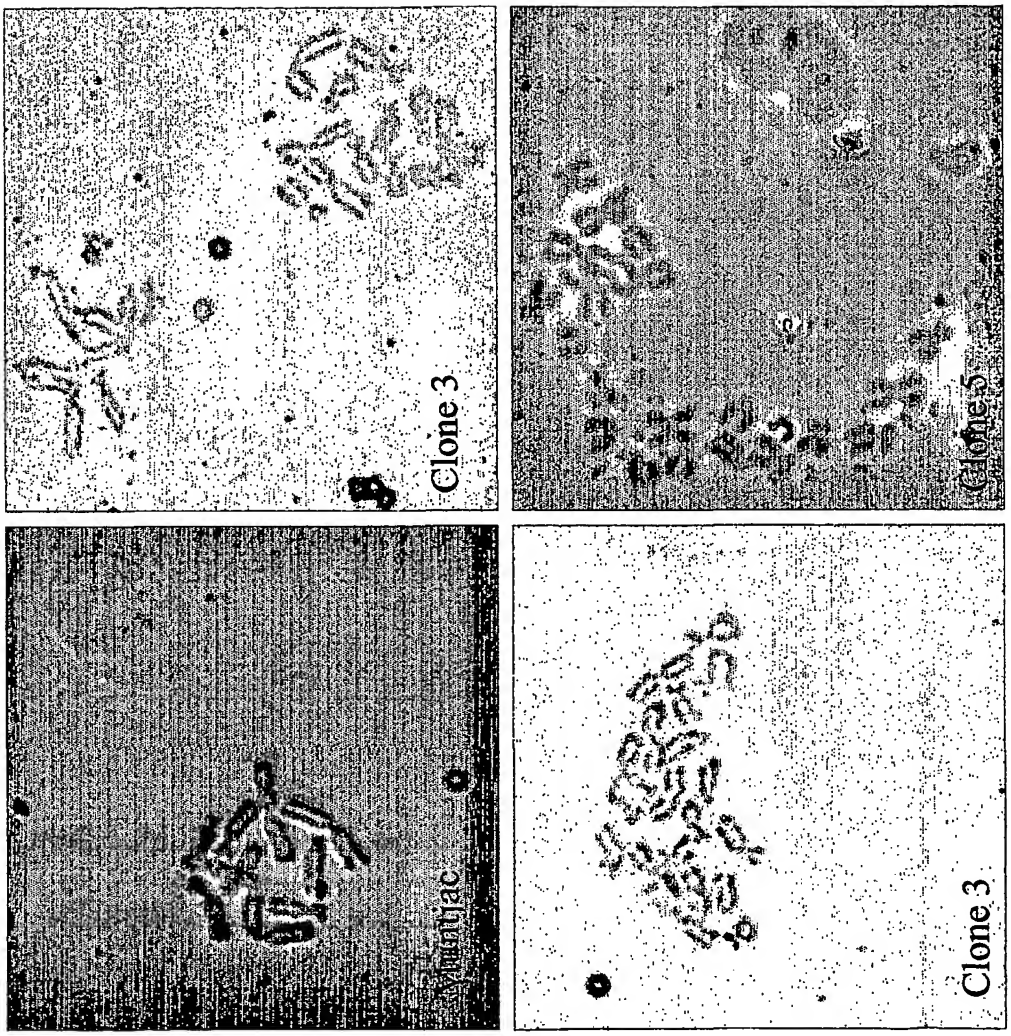


Fig. 7. Morphological analysis of reprogrammed sperm nuclei and muntjac cells

Fig. 8. Chromosome composition of muntjac & hybrid cells selected for drug resistance after fusion with human sperm nuclei



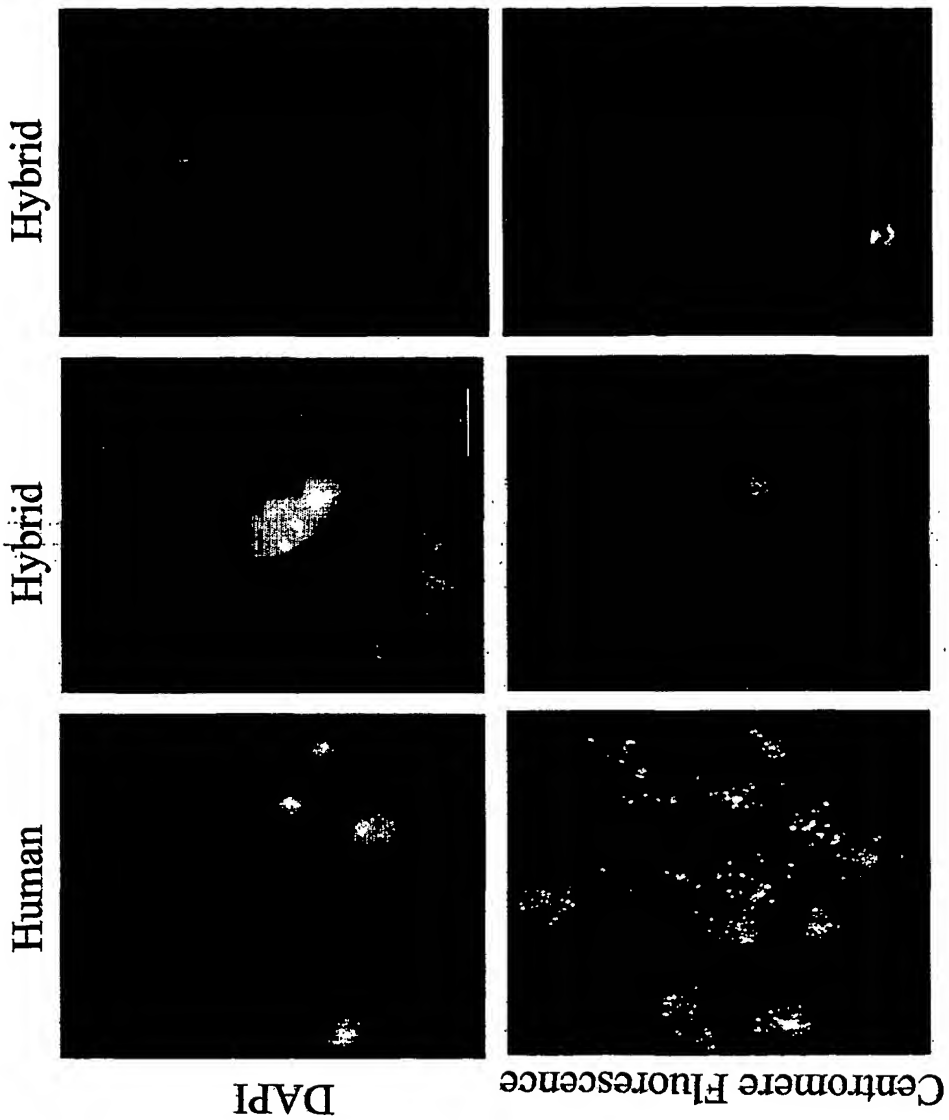


Fig. 9. Demonstrates human chromosomes in muntjac hybrid cells

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/024872 A3

(54) Title: MANIPULATION OF SPERMATOZOA

(57) Abstract: The present invention relates to a method of sperm permeabilisation comprising: (a) washing a sample containing sperm in a sperm nuclear extraction buffer (SNEB) which includes one or more metal chelating agents and which is substantially free of non-ionic detergents and divalent cations; (b) contacting the sperm with one or more permeabilising agents; and (c) optionally separating the sperm tail from the sperm head; and to a method of sperm transformation which method comprises incubating a sample of sperm or sperm nuclei in one or more cell-free extracts. Also described are extracts and buffers for use in these methods, methods of preparing the extracts and uses of the transformed sperm nuclei.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 01/04184

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 C12N5/02 C12N15/06 C12Q1/02 C12N13/00
A01K67/02 A61D19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, BIOTECHNOLOGY ABS, CHEM ABS Data, EMBASE, LIFESCIENCES, MEDLINE, PAJ, SCISEARCH, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 651 992 A (WANGH LAWRENCE J) 29 July 1997 (1997-07-29) column 4, line 17 - line 67 column 5, line 56 -column 6, line 27 column 7, line 56 - line 65 column 9, line 52 - line 63 column 11, line 20 -column 13, line 30 column 15, line 36 - line 40 column 21, paragraph 4 column 23, line 65 -column 26, line 49 example 13 ---	1-7, 9-18,27, 28
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 March 2002	15.04.02

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pilat, D

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COLLAS PHILIPPE ET AL: "Formation of the sea urchin male pronucleus in vitro: Membrane-independent chromatin decondensation and nuclear envelope-dependent nuclear swelling." MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 42, no. 1, 1995, pages 106-113, XP001034081 ISSN: 1040-452X cited in the application see Materials and methods section	1-7, 9-18, 25-27
X	LOHKA M J ET AL: "ROLES OF CYTOSOL AND CYTOPLASMIC PARTICLES IN NUCLEAR ENVELOPE ASSEMBLY AND SPERM PRONUCLEAR FORMATION IN CELL-FREE PREPARATIONS FROM AMPHIBIAN EGGS" JOURNAL OF CELL BIOLOGY, vol. 98, no. 4, 1984, pages 1222-1230, XP001037656 ISSN: 0021-9525 cited in the application see Materials and methods section the whole document	1-7, 9-18, 25-27
X	POCCIA D ET AL: "Transforming sperm nuclei into male pronuclei in vivo and in vitro." CURRENT TOPICS IN DEVELOPMENTAL BIOLOGY, vol. 34, 1996, pages 25-88, XP001038289 1996 Academic Press, Inc.;Academic Press Ltd. 1250 Sixth Ave., San Diego, California 92101, USA; 14 Belgrave Square, 24-28 Oval Road, London NW1 70X, England, UK ISBN: 0-12-153134-1 the whole document	1-7, 9-18, 25-27
X	GORDON K ET AL: "IN-VITRO ACTIVATION OF HUMAN SERUM INDUCED BY AMPHIBIAN XENOPUS-LAEVIS EGG EXTRACT" EXPERIMENTAL CELL RESEARCH, vol. 157, no. 2, 1985, pages 409-418, XP002194533 ISSN: 0014-4827 the whole document	1-7, 9-18,27

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 01/04184

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAWYER DENNIS E ET AL: "The use of an in vitro sperm activation assay to detect chemically induced damage of human sperm nuclei." REPRODUCTIVE TOXICOLOGY, vol. 9, no. 4, 1995, pages 351-357, XP002194534 ISSN: 0890-6238 abstract	7,9-17, 25,27,28
X	NEUBER EVELYN ET AL: "Efficient human sperm pronucleus formation and replication in Xenopus egg extracts." BIOLOGY OF REPRODUCTION, vol. 61, no. 4, October 1999 (1999-10), pages 912-920, XP002194535 ISSN: 0006-3363 the whole document	7,9-18, 25,27,28
X	KAWASAKI KATSUMI ET AL: "Chromatin decondensation in Drosophila embryo extracts." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 13, 1994, pages 10169-10176, XP002194536 ISSN: 0021-9258 the whole document	7-16,25, 27,28
T	LU PING ET AL: "Nuclear assembly of demembranated Xenopus sperm in plant cell-free extracts from Nicotiana ovules." EXPERIMENTAL CELL RESEARCH, vol. 270, no. 1, 15 October 2001 (2001-10-15), pages 96-101, XP002185219 ISSN: 0014-4827 see Materials and methods section	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 01/04184

B x I Observations where certain claims were found unsearchabl (Continuation of item 1 f first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

completely 1-18,22,27-28 partially 25,26

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: completely 1-6

   method of sperm permeabilisation comprising a) washing a sample containing sperm in a buffer which includes one or more metal chelating agents and is substantially free of non-ionic detergents and divalent cations and b) contacting the sperm with one or more permeabilising agents.

2. Claims: completely 7-18, 22, 27-28, partially 25,26

   method of sperm transformation comprising incubating a sample of sperm or sperm nuclei in one or more cell-free extracts, and sperm nucleus obtained therefrom.

3. Claims: completely 19, partially 25-26

   method of preparing a sperm dispersion extract from a cell sample comprising a) suspending the sample in a buffer substantially free of divalent cations b) centrifuging the sample and c) collecting the supernatant, and kit containing said cell-free dispersion extract.

4. Claims: completely 20-21

   method of preparing a sperm reprogramming extract comprising preparing a sperm dispersion extract from a cell sample comprising a) suspending the sample in a buffer substantially free of divalent cations b) centrifuging the sample, c) suspending the pellet product in a buffer and d) centrifuging the suspended pellet and f) collecting the supernatant product.

5. Claims: completely 23,24, partially 25-26

   method of testing a sample of sperm or sperm nuclei comprising incubating the sample in a cell-free dispersion extract, and kits therefor

6. Claims: completely 29-32

   method of in vitro fusion between a transformed sperm nucleus and a somatic cell or oocyte.

7. Claims: 33-35

   method of testing for sperm chromosome abnormalities

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

comprising contacting a transformed sperm nucleus with a somatic cell, causing the two nuclei to undergo fusion and analysis of the chromosome, kits therefor.

8. Claim : 36

method of in vitro fertilisation comprising microinjection of a transfused sperm nucleus into the cytoplasm of an oocyte.

9. Claims: 37-39

method of reprogramming a somatic cell comprising incubating said somatic cell in one or more cell-free extracts.

10. Claims: 40-44

recipient cell into which the reprogrammed nucleus of a diploid cell has been introduced and animal embryo prepared therewith.

INTERNATIONAL SEARCH REPORT

nformation on patent family members

Int[illegible]onal Application No
PCT/GB 01/04184

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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